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# CYTOTOXIC T LYMPHOCYTE INDUCTION BY SOLUBLE T-CELL RECEPTORS

by

JOHN FRANCIS KROWKA

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## Abstract

This thesis describes research investigating the role of antigen-specific inductive signals in cytotoxic T lymphocyte (CTL) responses. Two fundamental questions are addressed:

1. What are the mechanisms involved in the triggering of CTL responses? Are only antigen-nonspecific lymphokines involved or is there a role for antigen-specific helper factors (ASHF) in inductive events? Do secreted forms of helper T lymphocyte (T*h*) antigen-receptor molecules function as positive immunoregulatory signals for CTL precursors (CTLp)?

2. Are the same antigen-specific inductive signals able to induce both CTL and IgM responses or do they exhibit functional target-cell specificity?

In order to address the first question, it was essential to separate ASHF from antigen-nonspecific lymphokines such as Interleukin 2 (IL2) by antigen-affinity chromatography of supernatants from long-term T*h* lines. An established helper-dependent assay system has been modified to demonstrate the antigen-specificity and H-2 restriction properties of ASHF in the induction of thymocyte CTL precursors (CTLp). This unique system we have developed enables comparison of the ability of a T*h* population or its secreted products to trigger CTLp with its ability to specifically help other types of immune responses to either soluble or cell-surface antigens. Antigen specificity is demonstrated in the binding of ASHF molecules only to nominal antigen both during purification and in tests of its functional activity.

The requirement for spatial proximity of the determinants recognized by the CTLp and ASHF was examined. It was observed that CTLp induction occurred only when the determinants recognized by the CTLp and ASHF were on the same stimulator cells, thus

meeting the criteria for "linked recognition". This requirement may function *in vivo* to minimize the induction of "bystander" CTLp that include self-reactive CTLp.

The role of major histocompatibility complex (MHC)-encoded products in ASHF-mediated induction of CTLp has been examined. Previous work from the laboratory of E. Diener and others suggested that T*h* antigen receptors (T*h*AR) and MHC-encoded Ia molecules were subunits of a functionally complete ASHF. Dissociation of the ASHF subunits was accomplished by the chelation of calcium ($Ca^{++}$) in order to examine the roles of these subunits in lymphocyte induction.

The ASHF, purified from supernatants of the T cell line CHI by antigen-affinity chromatography in the presence of $Ca^{++}$, is defined as $Ca^{++}$-sufficient ASHF, while ASHF purified on antigen-affinity columns in the absence of $Ca^{++}$, is defined to be $Ca^{++}$-deficient. $Ca^{++}$-sufficient ASHF is not H-2 restricted (as defined by the phenotype of the ASHF-producing cells) in the recognition of nominal antigen or in its interactions with CTLp or adherent stimulator cells. In contrast, when the "complete" ($Ca^{++}$-sufficient) ASHF is functionally dissociated into subunits by removal of $Ca^{++}$, the "incomplete" antigen-specific subunit of ASHF ($Ca^{++}$-deficient ASHF) is still H-2-unrestricted in its ability to bind nominal antigen but requires products from syngeneic adherent cells in order to trigger CTLp. When adherent cells that are H-2-identical to the ASHF are provided in culture, the "incomplete" ASHF is able to trigger either syngeneic or allogeneic CTLp in an antigen-specific manner.

The most reasonable interpretation of these findings is that an H-2 restricted molecular interaction occurs in CTLp induction by ASHF. An antigen-specific T*h*-derived receptor appears to require association with $Ca^{++}$ and self MHC-encoded molecules derived from adherent cells in order to form a "complete" ASHF that is able to trigger CTLp in an apparently H-2-unrestricted manner. It was demonstrated by ELISA and by binding of antigen-specific helper activity to a colum consisting of Sepharose-coupled anti-(T cell receptor) monoclonal antibodies that ASHF contains a secreted or at least "shed" form of T*h* receptors.

It is suggested that multimolecular complexes of antigen, T*h*AR, and Ia molecules constitute an inductive signal for CTLp. Ia antigens may function as a polymorphic glue to make the antigenically-monovalent T*h*AR functionally multivalent. Additionally, they are suggested to serve as structures that interact with complementary molecules on CTLp and the precursors of other classes of lymphocytes to deliver inductive signals.

Traditionally, the types of antigens used experimentally to induce CTL or antibody responses are very different from each other. However, the unique "hapten-carrier" system we developed, enabled us to approach our second fundamental question. We tested ASHF preparations for their ability to help CTL- or IgM-responses *in vitro*.

The supernatant of a non-cloned T*h* line (CHI) was found to contain two functionally and biochemically distinct types of ASHF that are separable by anion-exchange chromatography: one factor triggers CTL responses (ASHF-*CTL*), the other triggers IgM B cell responses (ASHF-*B*). Clone 4C6, derived from the parent line CHI, produces ASHF-*CTL* but not ASHF-*B*. These observations could result from either two completely distinct ASHF isotypes, or from the differential assembly of a common antigen-specific receptor with different antigen-nonspecific subunits to make AHF-*CTL* or ASHF-*B*. The antigen-nonspecific component(s) of ASHF may function to determine the class of immune response induced. It is suggested that T*h*AR in association with MHC-encoded products, constitute a product able to deliver primary inductive signals for both cell-mediated and antibody-mediated immune responses, prior to the effects of antigen-nonspecific lymphokines that mediate maturation and clonal expansion.

## Preface

The experiments in Chapters III and IV were done in collaboration with Drs. C. Shiozawa, V. Paetkau, E. Diener, and T. Mosmann. Some of these results have been published in the *Journal of Immunology* (Vol. 133:2018) and will be published in a volume entitled *Recognition and Regulation in Cell-Mediated Immunity* (Academic Press, Inc.). The results in Chapter IV have been submitted for publication. Drs. T. Mosmann and B. Singh collaborated in some of the experiments presented in Chapter V.

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# List of Abbreviations

| | |
|---|---|
| **AFC** | Antibody-forming cells |
| **APC** | Antigen-presenting cells |
| **ARS** | Azobenzenarsonate |
| **ASHF** | Antigen-specific helper factors - *CTL* or *B* refer to the ability to help CTL or IgM responses, respectively |
| **B** | Bone marrow-derived (lymphocytes) |
| ***B*** | B locus - the chicken MHC |
| **B10** | C57Bl10/J mice |
| **BCDF$\gamma$** | B cell differentiation factor for IgG |
| **BCGF** | B cell growth factor |
| **BRBC** | Burro red blood cells |
| **C** | Constant regions |
| **C'** | Complement |
| **$Ca^{++}$** | Calcium |
| **CHI** | A T*h* line that recognizes determinants on *B*2 CRBC |
| **Con A** | Concanavalin A |
| **CP** | *Corynebacterium parvum* |
| **CRBC** | Chicken red blood cells |
| **CTDF** | CTL-differentiation factors |
| **CTL** | Cytotoxic T lymphocytes |
| **CTLp** | CTL precursors |
| **CTR** | Cytotoxic T lymphocyte antigen receptors |
| **DTH** | Delayed-type hypersensitivity |
| **EGTA** | Ethylene glycol-bis-($\beta$-aminoethyl ether) N,N'-tetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **FCS** | Fetal calf serum |

| | |
|---|---|
| FTA | Freeze-thaw antigen |
| GVHR | Graft-versus-host responses |
| H-2 | The murine major histocompatibility complex |
| HY | Male histocompatibility antigens |
| IL1 | Interleukin 1 |
| IL2 | Interleukin 2 |
| IL3 | Interleukin 3 |
| Kd | Kilodaltons |
| KLH | Keyhole limpet hemocyanin |
| LD | Lymphocyte-defined MHC antigens |
| LPS | Lipopolysaccharide |
| MEM | Minimal essential medium |
| mH | Minor histocompatibility antigens |
| MHC | Major histocompatibility complex |
| MLR | Mixed lymphocyte reaction |
| Mls | M-(histocompatibility)-locus |
| MØ | Macrophage |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyl tetrazolium bromide |
| NK | Natural killer (cells) |
| NR | Non-rosette-forming cells |
| OVA | Ovalbumin |
| PBS | Phosphate buffered saline |
| PMA | Phorbol-12-myristate-13-acetate |
| PNA | Peanut agglutinin |
| PPD | Purified-protein derivative of tuberculin |
| RFC | Rosette-forming cells |

| | |
|---|---|
| **SD** | Serologically-defined MHC antigens |
| **sIg$^{-}$** | Negative for the expression of surface immunoglobulin |
| **SN** | Supernatant |
| **SRBC** | Sheep red blood cells |
| **T** | Thymus-derived (lymphocytes) |
| **TGAL** | Poly-L-(tyrosine, glutamic acid)-poly-DL-alanine- poly-L-lysine |
| **T*h*** | Helper T lymphocytes |
| **T*h*AR** | Helper T lymphocyte antigen receptors |
| **TNP** | Tri-nitro-phenol |
| **T*s*** | Suppressor T lymphocytes |
| **V** | Variable regions |
| **V*h*** | Variable region of immunoglobulin heavy chains |
| $^{+}$ | Positive for cell-surface expression |
| $^{-}$ | Negative for cell-surface expression |

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# I. INTRODUCTION

The immune system in vertebrates has evolved to provide defenses against pathogenic microorganisms and antigenically-aberrant autologous cells. In order to accomplish this goal while minimizing damage to healthy cells of the body, biological mechanisms have evolved that enable it to respond specifically to almost any foreign material. In addition to specfic recognition of antigens, the system provides a variety of cell-mediated and antibody-mediated cytotoxic effector mechanisms, each adapted for the elimination of different types of antigenic stimuli.

In order to achieve immune responses that are efficient at eliminating a given antigenic challenge, a complex network of collaborative interactions between functionally distinct cell types has developed. This includes collaboration by direct cell-to-cell contact and via secreted immunoregulatory molecules. During the past decade, much progress has been made in the definition of these interactions. However, many uncertainties remain regarding mechanisms employed for the induction and regulation of immune responses.

The primary focus of this chapter will be to review the mechanisms of cytotoxic T lymphocyte (CTL) induction in comparison to the mechanisms involved in the induction of other classes of immune responses in order to provide an integrated view of the phenomenology regarding the induction of antigen-specific immune responses. The role of soluble helper T lymphocyte antigen-receptor (T*h*AR) moities, i.e. antigen-specific helper factors (ASHF), as positive immunoregulatory signals will be discussed in detail.

## A. Cellular Collaboration in the Induction of Antibody Responses

Thymus-derived (T) lymphocytes play a central role in the induction and regulation of both antibody-mediated and cell-mediated immune responses to most antigens. A requirement for collaboration between functionally distinct cell types was first demonstrated in the induction of antibody responses. In adoptive transfer experiments, it was first demonstrated that T lymphocytes are required to "help" the induction of antibody responses to sheep red blood cells (SRBC) by bone-marrow derived (B) lymphocytes (1,2,3). This collaboration was defined more precisely by the experiments of Mosier *et al.* (4,5), that demonstrated a requirement for at least one type of plastic-adherent cells (macrophage-like cells) and two nonadherent cell types (B and T lymphocytes) in the induction of antibody responses.

Antigens such as SRBC were useful in demonstrating cellular collaboration but the complexity of their antigenic structure made difficult the precise analysis of the determinants recognized by the collaborating cell types. The conjugation of relatively small chemical groups (haptens) onto larger protein molcules or synthetic polypeptides (carriers), using methods developed by Landsteiner (6), provided a more defined type of antigen that could be manipulated for the dissection of cellular interactions in immune responses.

In the late 1950's Benacerraf and his colleagues began investigating immune responses to hapten-carrier conjugates (7,8) and later demonstrated that secondary anti-hapten responses were generated only when the same hapten-carrier conjugate was used in the primary and secondary sensitizations (9). However, the nature of collaborative interactions was not appreciated at this time.

The first direct evidence for collaboration between cells of distinct antigenic specificities was provided by Rajewsky in studies of antibody responses to lactic dehydrogenase isoenzymes (10). This was confirmed by Mitchison in an adoptive transfer system using hapten-carrier conjugates (11). Subsequently Raff demonstrated by treatment of sensitized spleen cell populations with anti-theta serum that the carrier-specific helper cells were thymus-derived and that the hapten-specific antibody-forming cell (AFC) precursors were not (12).

The concept of collaboration between distinct cell types recognizing carrier and haptenic determinants via an antigen bridge was further clarified by the experiments of Rajewsky (10,13) and Mitchison (14). Using hapten-reactive AFC precursors and carrier-reactive helper cells of distinct specificities, Mitchison demonstrated "a requirement for physical union of the hapten and carrier " in the induction of antibody responses (15), thus confirming Rajewsky's earlier observations (10). This requirement for "linked recognition" may provide a physiological safeguard that minimizes the potentially dangerous induction of self-reactive B lymphocytes or of those B lymphocytes which are not relevant to the elimination of a particular antigenic challenge. This requirement provides for the prevention of the induction of these "bystander" lymphocytes by antigen-nonspecific "long-range" signals. The investigations of Fong *et al.* (16) defined the maximum distance between hapten and carrier determinants to be 69-97 angstroms for triggering of hapten-reactive AFC by carrier-reactive helper T lymphocytes (T*h*). The biochemical basis for this phenomena, however, has not been defined. Recently, the term "cognate interaction" has been introduced to define a requirement for recognition by T*h* of carrier determinants associated with major histocompatibility complex (MHC)-encoded molecules in addition to the requirement for hapten-carrier linkage (17).

Studies by Lake and Mitchison (18) demonstrated that independent molecules on a cell-membrane surface were able to associate in such a manner as to form an "intrastructurally- (as opposed to intramolecularly) linked" immunogenic complex recognized by T*h* and B lymphocytes. Viral antigens recognized by T*h* are also able to form intrastructurally-linked associations with Thy-1 molecules for the triggering of antibody responses (19). Not all molecules on the surface of the same cell, however, are able to associate in such a manner (20). Preferential association may bring functionally related molecules in close apposition for collaborative interactions with other cells (21-23).

A number of studies indicate that the concentration of antigen is critically important for the demonstration of a requirement for linked recognition in the experimental induction of antibody responses by cloned T*h* cell lines or hybridomas (24-27). High concentrations of carrier are able to bypass the requirement for physical linkage of hapten and carrier (25,27). It has been suggested that high antigen concentrations stimulate production by T*h* of lymphokines that are antigen-nonspecific and genetically unrestricted (25). In contrast, lower concentrations of antigen may stimulate the same or different types of T*h* to produce a qualitatively different type of help such as antigen-specific helper factors (ASHF), that require cognate interactions in order to trigger lymphocyte responses.

A growing body of evidence suggests that the requirement for linked recognition may be common to the induction of both cell-mediated and antibody-mediated immune responses. A requirement for "linked recognition" has been demonstrated in the induction of delayed-type hypersensitivity (DTH) effector T lymphocytes (28). There also exists a requirement for linked recogntition in the generation of T*h* that participate in the induction of DTH effector cells (P. Bretscher, personal communication). Evidence suggesting a role for "linked recognition" in the induction of CTL responses has been reviewed recently (29), and will be discussed in section IE of this thesis.

These early experiments defined the framework for collaboration among T*h*, adherent cells, and the precursors of AFC. The "two signal" model of antibody induction suggested that in addition to antigen (signal one), an antigen-specific T cell-derived inductive signal was required for immune responses to most antigens (30).

A wide variety of experiments illustrated and clarified the critically important role of MHC products in the generation of both antibody and cell-mediated immune responses (31). Genetic, functional, and biochemical experiments demonstrated that T*h* can be activated by nominal (foreign,non-MHC) antigens in association with MHC products on the surfaces of macrophages and macrophage-like antigen-presenting cells (APC) (32-36). Recent evidence suggests that murine or human B lymphocyte tumor cells, EBV-activated cells, or lipopolysaccharide (LPS)-induced blast cells are able to present antigens to T*h* (37-41) but the physiological relevance of this type of antigen presentation is unclear. It is also unclear how T*h* activation, defined solely by antigen-specific proliferation or IL2 release in many experimental systems, correlates with the ability of a given T*h* population to help cell-mediated or antibody responses.

The activation of T*h* results in their ability to deliver inductive signals to AFC precursors or to the precursors of cell-mediated immune responses. The recent identification and analysis of the $\alpha$ and $\beta$ subunits of T lymphocyte antigen receptor genes and glycoproteins (42-46) should provide a more precise definition of requirements for antigen binding and activation of T*h* by APC. Release of Interleukin 1 (IL1) and possibly other cytokines by APC plays an important role in the activation of T*h* (47,48).

It has been demonstrated that T*h* induction requires collaborative interactions with other helper cells in order to induce antibody responses (48), DTH responses (P. Bretscher, personal communication), or CTL responses (Ref. 29 and Pilarski *et al.*, manuscript in preparation). Experimental evidence indicates that both antigen-specific and antigen-nonspecific helper factors such as IL2, secreted by T*h* subsets are able to provide help directly to themselves, other T*h*, and/or to functionally distinct effector cell precursors (17,50-56). Stimulation of APC by T*h* products such as Interleukin 3, γ-interferon, and other lymphokines may function as indirect routes of T*h* autostimulation (56,57).

It is unclear how quantitative or qualitative variations in the presentation of antigen by APC, in the recognition of antigen by T*h* or in the delivery of T*h*-inductive signals by APC or other T*h* may influence the induction of a given T*h*. Different types of APC may stimulate antigen-specific proliferation but differ in their ability to preferentially induce T*h* or suppressor T lymphocytes (T*s*) (58). A growing body of evidence indicates that proliferation of T cell clones is highly dependent on antigen concentration and that specific proliferation, IL2 release, and the ability to help antibody responses may be independently inducible (59-61). The role of multiple and functionally distinct subsets or maturational stages of T*h* that differ in their activation requirements and/or the type of inductive stimuli they are able to deliver upon activation remains to be resolved.

The roles of direct cell-cell contact, secreted forms of T*h* antigen receptors, and antigen-nonspecific lymphokines in cooperative interactions between T*h* and AFC precursors or the precursors of cell-mediated immune responses require further elucidation. Some evidence exists to support the hypothesis that distinct subsets of AFC precursors may differ in the type of signals they require for activation (62,63). Analogously, distinct subsets of precursors for cell-mediated immune responses may differ in their activation requirements. It has been suggested that different classes of immune responses require different numbers of helper signals for induction, DTH responses requiring fewer than IgG, for example (64). Antigen-nonspecific lymphokines appear to differ qualitatively in the class of immune response they preferentially induce by virtue of their target cell specificity (65). Recent evidence indicates that the antigen-nonspecific lymphokine, B cell differentiation factor for IgG (BCDF$\gamma$) preferentially induces IgG$_1$ secretion in a subset of surface immunoglobulin negative (sIg$^-$) B cells which are precommitted to IgG$_1$ secretion (66,67).

Currently, many laboratories are investigating the role of MHC-encoded products and of soluble mediators in inductive events. However, even today the precise nature and sequence of these events remain to be defined in physiologically relevant terms. The complexity of collaborative interactions in the induction of antibody responses is presently a subject of great controversy (63-68), which is beyond the scope of this thesis.

### B. Collaboration in Cell-Mediated Immune Responses

Transfer of DTH or allograft reactivity (69,70) and anti-tumor immunity (71,72) by lymphoid cells but not by serum from immune animals distinguished cell-mediated from antibody mediated immune responses. The demonstrations that neonatal thymectomy greatly reduces the ability to mount DTH responses or to reject allogeneic cells suggested that thymus-derived cells play an important role in cell-mediated immune responses (73). However, the interactions among cell types involved in these reactions were not clear. In order to define the functions of cells participating in these *in vivo* responses, experimental systems demonstrating antigen-specific cellular reactivity *in vitro* and *in vivo* were developed.

The migration inhibition test was developed as a correlate of DTH reactivity (74,75). Peritoneal exudate cells from guinea pigs sensitized to purified protein derivative of tuberculin (PPD) or ovalbumin were centrifuged in capillary tubes and cultured at 37°C (75). Only in the presence of the sensitizing antigen was the cellular migration out of the tubes significantly inhibited. This suggested a mechanism for localizing cells at an inflammatory site. This study and others demonstrated that migration was specifically inhibited by cells from DTH-reactive animals but not by those animals producing circulating antibodies to the sensitizing antigen (75-77).

The specific *in vitro* inhibition of migration by cells from animals sensitized to hapten-carrier conjugates demonstrated specificity for the carrier (77,78), similar to results reported for secondary antibody responses (9). Specific inhibiton of migration has also been reported when peritoneal cells from animals that had rejected a skin graft were cultured with lymphoid cells from mice of the same strain as the skin graft donor (79). However, collaboration among cell types participating in these responses was not identified. Furthermore, it is not clear if migration-inhibitory activity is an essential correlate of *in vivo* DTH reactivity or if it is merely a coincidental phenomenon. More recent *in vitro* studies have established a requirement for T*h* in the induction of DTH responses and have begun to elucidate the collaborative interactions in the generation of these responses (28,80,81).

Another *in vitro* technqiue, the mixed lymphocyte reaction (MLR) was also developed during the 1960's to quantitate specific cellular proliferation in response to allogeneic cells (82-84). By mixing human or murine lymphocytes from unrelated individuals *in vitro*, strong proliferative responses could be generated. In the one-way MLR, inactivation of the proliferative ability of the "stimulator" population by mitomycin-C treatment or by $\gamma$- or X-irradiation allowed quantitation of "responder" cell proliferation (85). Subsequently, synergism between anatomically distinct populations of murine thymus-derived cells in the MLR was demonstrated (86).

Synergy between different types of T lymphocytes was first demonstrated in graft-versus-host responses (GVHR) (87). When two anatomically distinct lymphoid populations such as peripheral blood lymphocytes and thymocytes from one of the MHC-incompatible parental type mice were inoculated into neonatal F1 recipients, the magnitude of GVHR was significantly greater than the sum of the responses by either population alone. Substitution of F1 thymocytes or peripheral blood lymphocytes for parental cells failed to result in synergy, indicating that both populations require recognition of foreign antigens for collaboration to occur.

Depletion of GVHR activity by neonatal thymectomy of parental strain donor mice (87) or by anti-theta and complement treatment of parental strain lymphoid cell populations (88) demonstrated that GVHR were mediated by T cells. Differences between the collaborating populations were observed in their sensitivity to anti-thymocyte serum (87). These and subsequent studies (89,90) confirmed collaboration in GVHR betweeen functionally-distinct T lymphocyte subpopulations. However, a number of T cell types may be activated in GVHR (91) and it was necessary to utilize *in vitro* systems for the dissection of collaborative events in the induction of T cell responses.

## C. Cellular Collaboration in the Induction of CTL Precursors

In order to identify the cells participating in tissue-destructive reactions such as rejection of allogeneic grafts, DTH responses, or the rejection of tumor cells, *in vitro* tests were developed to demonstrate the specific cytotoxic activity of lymphoid cells in the absence of antibodies or complement. Although Medawar and others had unsuccessfully attempted to demonstrate cell-mediated cytotoxic activity even from appropriately immunized animals (92,93), Govaerts was able to provide the first evidence for specific cell-mediated cytotoxicity by lymphoid cells *in vitro* (94). In this report, thoracic duct lymphocytes from a dog that had received a kidney graft were cytotoxic to cells from the donor but not to cells from an unrelated dog. This and subsequent reports in other systems, reviewed elswhere (95), confirmed a specific contact-dependent, serum-independent cytotoxic activity of lymphoid cells from animals sensitized *in vivo* with allogeneic normal cells, tumor cells, or skin grafts.

The development of a rapid quantitative assay for cyotoxicity, the $^{51}$Chromium-release assay (96,97), greatly facilitated the analysis of these reponses. Depletion of cytotoxic activity by treatment of the effector cells with anti-theta serum demonstrated that the cells responsible were thymus-derived and distinguished this activity from antibody-dependent cellular cytotoxicity and macrophage-mediated killing (98,99).

By analysis of the requirements for the generation of antigen-specific cytotoxic activity in a one-way MLR, it became possible to identify the requirements for induction of CTLp as well as the effector functions of CTL. Using spleen cells or thymocytes from unimmunized mice as responder cells, Wagner and his colleagues were able to demonstrate in an MLR, the generation of antigen-specific, complement-independent cytotoxicity by theta-positive cells (100-102). Subsequently, synergism between thymocytes and lymph node cells or peripheral blood lymphocytes in the generation of CTL activity was demonstrated in the MLR (86,103). In a comparison of the proliferative and CTL activities generated in the MLR it was demonstrated that high numbers of thymocyte responder cells that were optimal for proliferative activity were weakly cytotoxic, suggesting that these two responses are dissociable (86). In these experiments it was suggested that thymocytes acted mainly as helper cells, similar to their apparent role in the induction of GVHR (87).

Antisera that recognize murine lymphocyte-surface alloantigens, developed by Boyse and his colleagues (104,105), provided a method for distinguishing between the following T cell subsets: a) Ly-1,2,3$^+$ b) Ly-1$^+$2,3$^-$ and c) Ly-1$^-$2,3$^+$ cells. The studies of Cantor and Boyse (106) provided the first evidence for functional differences between these subsets. In these studies it was shown that all three subsets proliferate in the MLR but CTL activity develops from the Ly-1$^-$2,3$^+$ T cells but not from the Ly-1$^+$ populations. The helper function of the Ly-1$^+$ subset was demonstrated in its ability to enable the generation of a vigorous AFC response to SRBC in an adoptive transfer system. Subsequent studies provided the first evidence for synergistic interaction between Ly-1$^+$ "amplifier" cells and Ly-2,3$^+$ CTLp in the generation of CTL activity in response to allogeneic stimulator cells *in vitro* (107).

It was suggested by a number of investigators (107-111) that in response to H-2-incompatible stimulator cells, Ly-1$^+$2,3$^-$ cells (as defined by their sensitivity to anti-Ly-1 antiserum and complement) are helper cells that recognize Ia antigens (also called Class II MHC antigens - 111). Based on studies using recombinant mouse strains which differed in defined subregions of their MHC, it was suggested that T*h* that reacted to "lymphocyte defined" (LD) antigens (Class II antigens) triggered CTLp recognizing "serologically defined" (SD) (Class I) MHC antigens (109,111).

One must not make assumptions, however, regarding the antigen-specificity or function of T cell subsets or clones merely on the basis of their Ly-1,2,3 phenotype. It has also been demonstrated in the induction of CTL responses that at least some types of T*h* can respond to allogeneic Class I MHC antigens, M-locus (Mls) alloantigens, or to nominal antigens in association with Class I or Class II MHC molecules (111-118). Ly-$1^-2,3^+$ cells can act as helper cells (119) and Ly-$1^+2,3^-$ cells (120,121) or Ly-$1,2,3^+$ T cells (122,123) can function as CTL. Also, Ly-$1^+2,3^-$ cells have been demonstrated to mediate DTH responses (80,121) Although the function of the Ly-1 molecule is unknown, it has been suggested recently that it may function as a sensor of positive immunoregulatory signals (29). Due to the multiplicity of functions mediated by Ly-$1^+$ T lymphocytes, the Ly-7 antigen, demonstrated to be selectively expressed on T*h* effectors participating in CTL induction (124), merits further attention as a useful marker for identifying T*h* subsets.

A more precise definition of the collaboration between T*h* and CTLp was provided by experiments demonstrating a requirement for T*h* in response to allogeneic stimulator cells (125,126), virally-infected cells (116,127,128), minor histocompatibility antigens (mH) (129), or trinitrophenylated syngeneic cells (130). In the culture system developed by Pilarski (125), an absolute requirement for antigen-specific radioresistant T*h* in the *in vitro* induction of CTL from low numbers of thymocyte CTLp was demonstrated. These T*h* were demonstrated to be able to collaborate with both syngeneic and allogeneic thymic or splenic CTLp (125,127). It is likely that the T*h* in these systems triggered CTL responses both by direct collaboration with CTLp and with T*h* precursors in the responder population.

In addition to the requirements for T*h* and CTLp, it was demonstrated that macrophage-like cells are required for the induction of CTL responses to allogeneic cells (131,132), tumor cells (133), or trinitrophenylated syngeneic cells or proteins (134,135). Distinct subsets of macrophages or dendritic cells which differ in their size, expression of Ia antigens, production of IL1, or their state of activation exhibit differences in their ability to induce CTL and other types of responses (136-138).

The studies of Weinberger *et al.* further defined the phenotypic characteristics and the antigen processing and presenting functions of the adherent cells required for the induction of CTLp (139-142). By depleting splenic adherent cells from both responder and stimulator populations, they demonstrated a requirement for $Ia^+$, Thy $1.2^-$, and radiation resistant adherent cells for the induction of primary and at limiting antigen dose, secondary CTL responses to irradiated H-2-incompatible stimulator cells. Adherent cells were also required for secondary responses to allogeneic plasma membranes or purified H-2K antigens in liposomes. In addition to the effects of adherent cells on T*h*, studies using cloned CTL lines demonstrated that APC may directly amplify the response of CTLp to IL2 (143).

These requirements for cellular collaboration in the induction of CTL responses exhibit a great deal of similarity to those of antibody responses. Both types require the participation of T*h*, macrophage-like cells, and the precursors of effector cells. Beyond this basic similarity, large gaps remain in our knowledge of common and distinctive mechanisms involved in the generation of CTL or antibody responses. Details of the interactions between T*h* and APC and between T*h* and CTLp will be discussed subsequently.

## D. Molecular Aspects of Interactions Between Helper T Lymphocytes and APC in the Induction of CTL Responses

The primary antigen-specific interaction in the generation of immune responses, including CTL responses, involves binding of antigen to the clonally-expressed T*h* receptors. Genetic and biochemical analyses have identified the basic T*h*AR unit expressed on cloned T cell lines and hybridomas that specifically secrete IL2 and/or proliferate in response to antigen (42-46,144-146). This 90 kilodalton (Kd) heterodimer consists of a slightly heavier, acidic $\alpha$ chain and a weakly basic $\beta$ chain that are linked by a disulfide bond. The T*h*AR heterodimers are expressed on the cell surface at a concentration of approximately 25,000 to 30,000 molecules per cell (144-146).

Peptide analysis of T*h* $\alpha$ chains indicate that they contain both variable (V) and constant (C) regions (147,148). However, identification of the mechanisms of gene rearrangement that generate V regions of the $\alpha$ chain of T*h* must await the reports of cloning of these genes. It is not known if $\alpha$ chains differ among T*h*, T*s*, and CTL.

The $\beta$ chain has been mapped to chromosome 6 of the mouse and chromosome 7 in humans, demonstrating that it is not linked to genes of the immunoglobulin heavy chain or the MHC (149). If the reports of T*h*AR C regions linked to the immunoglobulin heavy chain locus are correct (150), they must be located on the $\alpha$ chain, which has not been mapped. Genes encoding both V and C regions of $\beta$ chains have been identified (41-45,151-153). Comparison of $\beta$ chains of T*h* and CTL indicate they both use the same C regions (45).

Analysis of the genes encoding $\beta$ chains of T*h* and tumor cells has revealed that gene rearrangement mechanisms, similar to those used for immunoglobulin production, are employed to create a diverse repertoire for antigen recognition (42-46,151-154). Comparison of a limited number of $\beta$ chain V regions does not show any correlation between sequence and H-2-restriction properties of the clone from which they were derived (M. Davis, personal communication).

Rearranged genes encoding variable regions are spliced together with constant region exons to form complete $\beta$ chain genes. Two nearly identical exons encoding C regions of the $\beta$ chain have been identified (46,153) but there may also exist different C regions of the $\beta$ chain that have not been detected under the conditions employed for hybridization. Experiments by Malissen *et al.* (153) demonstrate that The C$\beta$2 gene has four exons encoding external, transmembrane, and cytoplasmic regions. It is not known if alternative splicing of transcripts of constant region exons, similar to the mechanisms used to generate secreted or membrane forms of immunoglobulins (155-157), may be employed to generate these distinct species of $\beta$ chains. Secreted forms of T*h*AR $\alpha$ and/or $\beta$ chains may be generated by differential post-translational modifications such as cleavage of transmembrane segments and/or by glycosylation. Secreted forms of T*h*AR $\alpha$ and $\beta$ chains may be used to enable T*h*AR to function as soluble immunoregulatory molecules, as will be discussed further in the following sections of this thesis.

Haskins *et al.* have described a monoclonal antibody (KJ16-133) that appeared to recognize C region allotypic determinants (158). The determinant recognized by KJ16-133 is expressed on some CTL and T*h* hybridomas and does not correlate with functional activity, antigen-specificity, or MHC-restriction. Similar to immunoglobulin allotypes, KJ16-133 appears to exhibit allelic exclusion. More recent evidence suggests that KJ16-133 does not recognize either of the two $\beta$ chain C regions but may recognize a determinant encoded by V, diversity (D), or joining (J) regions (Drs. J. Kappler and M. Davis, personal communication). As the D region only encodes for three amino acids (154), it is unlikely that KJ16-133 is recognizing these determinants exclusively. As there appear to be only 7 J regions in the $\beta$ gene complex (153), the observed frequency of KJ16133-reactive cells in spleen or thymus (about 20%) (158) suggests that this monoclonal antibody may be recognizing a determinant encoded at least partially by a J-region. It is also possible that KJ16-133 recognizes a joint $\alpha/\beta$ determinant. Experiments presented in this thesis demonstrate that determinants recognized by KJ16-133 can be detected on soluble antigen-specific helper moities that are able to bind antigen in an apparently H-2-unrestricted manner.

It has been suggested that T*h* or CTL antigen-receptors cannot bind soluble antigens in the absence of MHC products (159,160). However, a number of reports argue againtst this hypothesis. Carel *et al.* (161) demonstrated that haptenated cytochrome *c* peptides were specifically bound by Lyt$1^+2^-$ T cell clones. The studies of Rao *et al.* suggest that T*h* clones can specifically bind the azobenzenearsonate (ARS) hapten in the absence of APC and the MHC products they express (162,163). Binding and T*h* activation were specifically inhibited by structurally-related analogs of p-ARS. In the reports of Rao *et al.* (162,163), antigen-T*h* binding is necessary but appears not to be sufficient for T*h* activation.

It is important to distinguish binding of antigen to T*h*AR from T*h* activation. Bretscher and Cohn (30) have proposed that "immunological paralysis occurs when one antigenic determinant binds to one receptor ... on an antigen-sensitive cell, whereas induction requires the stretching of that receptor". Signal one may be either an inductive or a tolerogenic signal, depending on the presence or absence of signal two, respectively. Furthermore, binding of antigen to T*h*AR may occur in the absence of detectable T*h* function such as lymphokine release, proliferation, or the ability to help cell-mediated or antibody mediated immune responses. The studies of Rao *et al.* (162) demonstrate that 10 to 30 fold more antigen is required to stimulate IL2 production than DNA synthesis.

The studies of Lamb *et al* (164) provide a different approach that suggests MHC-independent binding of antigen. Preincubation of T*h* clones with relatively high concentrations of a synthetic hemagglutinin peptide in the absence of APC specifically abrogated the ability of influenza-specific T*h* to proliferate subsequently in response to nominal antigen and APC. The ability of IL2 to stimulate proliferation of these preincubated T*h* indicated that this specifically-unresponsive state was not due to toxic effects of the peptide. These T*h* remained specifically unresponsive for at least 7 days despite the presence of IL2 in the culture medium. Although the possibility of receptor blockade is not ruled out as a mechanism for the induction of this apparently tolerant state, these experiments suggest that T*h* can bind antigen in the absence of APC. All of these studies (161-164), however, fail to eliminate the possibility that passively-acquired or endogenously-expressed MHC restriction elements of T*h* clones enabled them to "present" antigen to each other for binding.

Cell-surface interaction molecules, including T*h*AR, enable collaboration by at least two possible mechanisms that may be sequential. Firstly, the induction of a conformational change or the appropriate clustering of membrane-bound T*h*AR by antigen in association with MHC-encoded molecules may result in the delivery of a functionally complete inductive signal. Secondly, multiple attachment sites provide the high avidity bonds to anchor T*h* and APC together for effective communication of hormone-like inductive signals including IL1, IL2, IL3, and γ-interferon, as discussed previously.

Clustering of receptor-ligand complexes has been implicated in the triggering of a wide variery of cellular functions by "hormones" such as epidermal growth factor and insulin (165-167). Clustering of IgE receptors on mast cells by divalent anti-IgE-receptor antibodies is able to trigger histamine release (168). A model has been proposed to suggest that clustering of membrane receptors generates cooperative interactions with other receptor molecules of the same type (169). Both clonally- and nonclonally-expressed molecules on T*h* may be required to interact with complementary molecules on APC for the delivery of inductive signals. If all required elements of a multimolecular T*h* receptor complex were not appropriately positioned, it is suggested that under physiological conditions T*h* or any other precursors would not receive an inductive signal.

Antigen-nonspecific receptor molecules in the T*h*AR complex may have passive and active functions in T*h* activation. They may function passively as a multivalent glue with acceptor sites for T*h*AR constant regions and for APC molecules. This may maintain contact between the molecules in a T*h* cluster and complementary APC molecules for a period of time sufficient for the delivery of an inductive signal. Interactions of these non-antigen-binding molecules in the T*h*AR complex with complementary structures on APC may induce conformational changes in T*h*AR or other molecules to potentiate activation.

Polymorphic Ia determinants on APC that interact with T*h*AR are critically important in T*h* activation (31-36,170). As we have suggested recently (29), this may be due to interactions of polymorphic Ia determinants with both variable and constant regions of T cell receptors.

The simultaneous binding to T*h* of antigen associated with MHC products would be expected to significantly increase the stability of the T*h*-APC interaction. If nominal antigen dissociated from the receptor complex, a weaker binding by interaction molecules could maintain T*h*-APC contact, at least temporarily. If the concentration of nominal antigen were sufficiently high, the dissociated nominal antigen would be quickly replaced and a more stable interaction of the antigen-MHC-T*h*AR complex would be reestablished.

A requirement for recognition by T*h* of nominal antigen in association with polymorphic determinants on syngeneic class II MHC molecules has been demonstrated in many experimental systems (23,25,31-41,60,160,170). It has been demonstrated that T*h* exist that recognize allogeneic MHC products in a MHC-unrestricted manner or recognize nominal antigens associated with syngeneic or allogeneic Class I or Class II MHC products (109-118,123,171). The T cells, possibly including T*h*, that proliferate in response to M locus (Mls) determinants in an MLR appear not to be H-2 restricted (172).

Recognition by T*h* of nominal antigen in association with MHC products can be viewed as being similar to the MHC-unrestricted recognition of allogeneic MHC products or other antigens. All of these types of interactions may fill sufficiently the binding cleft on T*h*AR to maintain contact for initiation of T*h* activation by APC. However, they may differ qualitatively and quantitatively in their ability to cluster molecules in a T*h*AR complex. These variations may be obscured in experimental systems by the generation of antigen-nonspecific lymphokines such as IL1 or IL2 that bypass a requirement for a functionally complete inductive signal.

In the induction of CTL responses to allogeneic MHC products, a requirement for syngeneic $Ia^+$ adherent APC has been demonstrated (142). However, if syngeneic-$Ia^+$ APC are depleted, allogeneic APC are able to trigger CTL responses (140). It is unclear if this is due to the direct interaction of APC with T*h*, CTLp, or both. Furthermore, it is unclear if distinct subsets of T*h* or CTLp, differing in their requirements for induction, are responding to $Ia^+$ or $Ia^-$ APC.

The studies of Elliot *et al.* (173-175) demonstrate that $Lyt1^+2^-$ responder cells in a MLR bind both stimulator- and syngeneic-Ia molecules. Antibodies recognizing responder Ia or V*h*-framework determinants strongly inhibited the binding of membrane vesicles containing stimulator Ia. Antibodies recognizing V*h* framework determinants also inhibited the uptake of syngeneic Ia. These results suggest the existence of self-Ia acceptor sites close to the antigen recognition sites on Lytl$^+$2$^-$ MLR blasts. Studies by Minami and Schreffler (176) also demonstrated that syngeneic IA$^+$ or IA/E$^+$ stimulator cells are required in primary, but not in secondary MLR against Class I MHC antigens.

Similar studies by Schnagl and Boyle (177) demonstrate that the precursors of cells that proliferate in response to MHC alloantigens will bind only to monolayers expressing both syngeneic and allogeneic MHC antigens (F1 cells), but not to monolayers of either parental type. It is unclear, however, if the proliferative cells in these systems are classical T*h*. The studies of Krzych *et al.* (178) suggest that DTH cells are the predominant proliferating cell type in responses of spleen cells to *β*-galactosidase.

These observations can be interpreted to suggest that in addition to interactions between polymorphic determinants on Ia antigens and T*h*AR V regions, interactions between polymorphic or conserved determinants on Ia antigens and C regions of T*h*AR molecules and/or other molecules in the T*h*AR complex are important in T*h* activation. These interactions may facilitate high-avidity binding and activation.

It has been suggested (29) that interactions between polymorphic Ia determinants and T*h*AR C regions would not be able to "adaptively differentiate" (179), unlike T cells expressing V region determinants that are selected according to the MHC type of the APC they encounter during antigen presentation. Analysis of antibody responses in responder/nonresponder radiation chimeras, allophenic mice, or neonatally tolerized mice can be interpreted to suggest the importance of these types of interactions (179-182). Effective triggering of T*h* and CTLp (183) may require interaction with self-Ia in addition to the H-2-restricted or unrestricted recognition of nominal antigen.

Cell-free complexes of Ia and processed antigen appear to be able to trigger T*h* in a genetically restricted and antigen-specific manner (184,185). It is not clear if the genetic requirement for I-region compatible APC in the triggering of T*h* by these soluble Ia-antigen complexes is due to interactions betweeen Ia and T*h*AR variable regions only or if interactions between polymorphic and/or nonpolymorphic Ia determinants with constant regions of T*h*AR or other T*h* interaction molecules are also important.

Nonpolymorphic determinants on Ia molecules may also function as interaction structures for T*h*-APC collaboration. The L3T4 molecule, which appears to be the murine analog of the human Leu3/OKT4, plays an important role in the binding and activation of T cells reactive with allogeneic Class II molecules or those reactive with nominal antigen associated with syngeneic Class II molecules (186,187). The ability of L3T4$^-$ variants to produce IL2 in response to the appropriate IA stimuli demonstrates that L3T4 is not essential for either antigen recognition or T*h* activation (187). It has been suggested that L3T4 facilitates T*h* activation by increasing the overall avidity of T*h*-APC interactions.

Although L3T4$^+$ T cells often recognize Class II molecules, an L3T4$^+$ T cell hybridoma (3DT52.5) that specifically recognizes the N and/or C1 domains on H-2$D^d$ (Class I) molecules has been reported (188). It is unclear whether this apparent exception to the rule is due to cross-reactivity between a H-2$D^d$ -N/C1 determinant and an unknown nominal antigen in association with Class II-MHC determinants. The demonstration of antigen-specific CTL activity and IL2 release by 3DT52.5 clearly illustrates that not all IL2-releasing cells are conventional T*h*. Monoclonal antibodies directed against stimulator IA determinants of the *b,d,* or *k* haplotypes are able to inhibit IL2-release by L3T4$^+$ 3DT52.5 in response to $D^d$ ,IA$^+$- but not to $D^d$ , IA$^-$-stimulators. L3T4$^-$ variants of 3DT52.5 are not inhibited by these antibodies. This evidence has been interpreted to suggest that L3T4 interacts with nonpolymorphic IA determinants to facilitate interaction of T cells with APC (186). However, an alternative explanation is that anti-IA antibodies interfere with the availability of the $D^d$ determinants required for recognition by 3DT52.5. Despite this objection, L3T4 appears to be an important T lymphocyte cell-surface molecule that participates in interactions with APC.

Other T*h* molecules including OKT3 in humans and Thy 1 in mice may be functionally important elements of an inductively-complete receptor cluster (55,144,161,189-191). Soluble clonotypic antibodies or antibodies recognizing the T3 determinant are able to inhibit antigen-specific proliferation and/or IL2 release (55,144,146). Soluble or Sepharose-linked anti-T3 or clonotypic antibodies augmented responses to IL2 by increasing the level of IL2 receptors (55,189). In contrast, Sepharose-coupled antibodies or appropriate stimulator cells but not soluble antibodies alone were able to induce IL2 release and subsequent proliferation. In this system Sepharose-coupled antibodies are not only a functionally complete inductive signal but they are able to stimulate the endogenous production by T*h* of IL2.

Studies by Kaye *et al.* (190) demonstrate that the induction of proliferation by soluble clonotypic antibodies required the presence of accessory cells (not MHC-restricted), IL1, or IL2. In the activation of T*h* by nanogram quantities of clonotypic antibodies, IL1 is required for expression of IL2 receptors (191). The soluble multivalent or divalent antibodies used in these experiments (55,144,189-191) presumably cluster molecules in the T cell receptor complex but this is qualitatively or quantitatively insufficient for complete activation. Apparently not all types of T*h*AR clustering are functionally identical.

Nonspecific lymphokines such as IL1 or IL2 may potentiate the aggregation of weakly-associated molecules in a recptor cluster. These types of triggering by antibodies recognizing molecules in the T*h*AR complex bypass requirements for antigen, Ia, or direct contact with APC. Inhibitors of nonspecific lymphokines that have been suggested to minimize this type of triggering *in vivo* (29,192) are apparently absent or at least ineffective in the *in vitro* culture systems used to demonstrate this potentiation of antibody-mediated T*h* induction. It is not clear if Sepharose-coupled antibodies or soluble antibodies plus nonspecific lymphokines induce other T*h* functions required to help cell-mediated or antibody-mediated immune responses, in addition to IL2 release or proliferation.

In addition to the relatively passive functions of the presentation of immunogenic molecular complexes to T*h*, some types of adherent cells are also capable of the active functions of antigen processing and the release of T*h*-inductive signals. Metabolic inactivation of stimulator cells by heat treatment, glutaraldehyde fixation, or ultraviolet (UV) irradiation, has been used to destroy these active functions for the analysis of their roles in CTLp induction.

Many reports demonstrate that priming is required for the generation of CTL from spleen cells cultured with metabolically inactivated lymphoid cells or fibroblasts (193-204). Normal spleen cells, in contrast, generate vigorous primary or secondary CTL responses to metabolically active ($\gamma$-irradiated) intact lymphoid stimulators. In order to generate CTL from unprimed spleen cells cultured with glutaraldehyde-fixed or UV-irradiated stimulators, the addition of an exogenous source of help such as antigen-specific helper cells (199), soluble helper factors including IL2 (52,197,202,203) or third-party allogeneic cells (109,204) are required. These studies demonstrate that metabolically-inactive stimulators are antigenically intact but are unable to deliver one or more of the signals required for the induction of T*h*, CTLp, or both.

Low numbers of thymocyte or splenic responders are also dependent on the addition of exogenous helper cells or their soluble factors to trigger CTL responses to metabolically-active stimulators (125,205-207). Metabolically-active stimulators are therefore unable to satisfy a requirement for T*h* in the induction of CTLp. In contrast, T*h* or their soluble products can bypass a requirement for metabolically-active stimulator cells (52,107,195,197,200-202). This suggests that metabolically active stimulators induce and augment the functions of T*h* in addition to their ability to express antigenic determinants recognized by CTLp. The demonstrations that UV-irradiation destroys the functions of APC required for the induction of antibody or DTH responses (208,209) may be due to similar defects in APC functions.

Many reports have documented the ability to generate weak primary- or strong secondary-CTL responses to cell-free allogeneic plasma membranes (132,139-141,203,210-215) or molecules incorporated into liposomes (139-142,216-218). A requirement for adherent cells was demonstrated in the induction of CTL responses to these subcellular antigens (132,139,215). This suggested that adherent cells were able to "present" constitutively-expressed or exogenously-added antigens. It was demonstrated that phorbol myristate acetate (PMA) is able to reconstitute the CTL response to adherent cells that were UV-irradiated after exposure to alloantigen containing liposomes but not to those

UV-irradiated before exposure to this antigen (139). This suggested that PMA could act as an inductive signal for T*h* but could not replace the antigen processing or presentation functions required for T*h* activation. In contrast, crude helper factors from mitogen-stimulated lymphocyte cultures were able to reconstitute responses to syngeneic adherent cells that were UV-irradiated before exposure to liposomes. This and other evidence has been interpreted to suggest that T*h* and CTLp exhibit different requirements for activation (140,219). The role of Ia molecules in the induction of CTLp will be discussed in the following sections.

Activation of T*h* is known to induce qualitative and quantitative changes at the levels of DNA synthesis and transcription, and at the level of mRNA translation into proteins (220). Changes in post-translational modifications, which are known to occur in normal B lymphocytes after induction (221) are also likely to occur as a result of T*h* activation.

Freeman *et al.* (220) examined the mRNA and proteins synthesized as a result of T*h* activation by Concanavalin A (Con A) or antigen. These studies demonstrate that *de novo* synthesis of mRNA and proteins, including IL2 and antigen-binding molecules was induced rapidly (within 15 hours) after activation. In contrast, proliferation proceeded at a much slower rate and did not reach optimal levels until approximately 48 hours after activation. At this time, IL2 production was negligible and the production of antigen-binding molecules, presumably T*h*AR, had decreased to about 30% of optimal levels. It appears that early mRNA species are shut off by 48-60 hours after activation and a different set of genes are then expressed.

T*h* activation does not appear to be an "all or none" process. The increase in IL2 receptor expression but not IL2 secretion by soluble anti-receptor complex antibodies suggests that some stimuli can induce some but not all of the requirements for activation (55,190,191). Do all types of T*h* respond in the same manner to these stimuli? Are their requirements for induction and the type of inductive stimuli they are able to provide to themselves, CTLp, and other cell types quantitatively or qualitatively different? Our data base is insufficient to do more than speculate at this point in time. Further comparative molecular and functional analyses of more T*h* clones and hybridomas is required.

### E. Inductive Signals for CTL Precursors

Activated antigen-specific T*h* or their secreted products are required for the induction of CTLp, as discussed previously. Similar to the mechanisms of interaction between APC and T*h*, T*h* may activate CTLp by clustering functionally related molecules in a CTLp receptor complex and by the delivery of hormone-like inductive signals.

The first step in in CTLp induction occurs at the level of antigen recognition by clonally-expressed CTL receptors. The CTL antigen receptor (CTR) exhibits a great deal of similarity to the T*h*AR (45,222). Both appear to use the same $\beta$ chain C regions and the same mechanisms of gene rearrangement (45,152,153). By quantitation of binding sites for clonotypic antibodies, it has been estimated that CTR are present on the cell membrane at a density of 30,000 to 40,000 per cell (144). It is likely that functional differences between CTR and T*h*AR reside on the $\alpha$ chain (45) although this has not yet been proven. [1] The selective pressures and/or the characteristics of CTR that favor recognition of Class I alloantigens or nominal antigens associated with syngeneic Class I antigens, in contrast to the predisposition of T*h*AR for recognition of allogeneic Class II antigens or nominal antigens associated with syngeneic Class II molecules remain to be elucidated.

The CTR function in the recognition of foreign molecules expressed on cell-membranes. Limiting dilution analysis has revealed a high frequency of CTLp reactive with foreign MHC alloantigens (206,223,224). CTL-precursors can also recognize nominal antigen ($X$) in the context of syngeneic or allogeneic MHC antigens, although the frequency of CTLp reactive with self-MHC+$X$ is much higher than that of CTLp reactive with allogeneic-MHC+$X$ (225-230). As predicted by Matzinger and Bevan (232), at least some of the CTL recognizing allo-MHC+$X$ can be explained by cross-reactivity with a different nominal antigen ($Y$) associated with self-MHC (self-MHC+$Y$) (232,232). Studies by Pilarski and Vergidis indicate that CTL can recognize minor histocompatibility antigens in an apparently H-2-unrestricted manner (215). Demonstration of cross-reactivity between self + *X* and allogeneic MHC determinants has been suggested to explain the high frequency of alloreactive CTLp (232-237) in terms of the "altered-self" model of CTL recognition, originally proposed by Pilarski (238). Studies by Hunig and Bevan (232), using cloned CTL lines, provide very strong evidence in support of this model.

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[1] Recent evidence suggests strongly that the cDNA clone reported by Saito *et al.* (45) does not in fact encode the $\alpha$ chain of the T cell antigen receptor (M. Davis, personal communication).

As discussed previously, T*h*-APC interactions and the recognition of nominal antigens by CTLp can be demonstrated to be MHC-restricted events in many experimental systems. In addition to H-2 restriction at the level of antigen recognition by V regions of T cell receptors, H-2 restriction may involve identical and nonidentical complementary molecules on T*h* and CTLp. Analysis of H-2-restricted interactions at the latter level is difficult to approach because antigen-nonspecific lymphokines such as IL2 may bypass or obscure the requirements for MHC products or for other molecules endogenously-expressed on T*h* or passively acquired by them. Our previous reports (29,239) and those of Corley *et al.* (183) indicate that polymorphic MHC determinants may be involved in collaboration between T*h* and CTLp. Nonspecific lymphokines may function *in vitro* to potentiate the effects of a weak and functionally incomplete interactions among antigen, MHC-encoded products, and the molecules in a CTR complex, in a manner analogous to their role in the potentiation of signals delivered to T*h* by soluble clonotypic antibodies (190,191).

Similar to results of studies on the role of MHC interactions between T*h* and B lymphocytes and/or APC (180-182), some studies suggest a role for polymorphic MHC determinants as interaction molecules in collaboration between T*h* and CTLp (183,240). The experiments of Corley *et al.* (183) clearly demonstrate a preference of primed T*h* for syngeneic CTLp and/or T*h* precursors. The experiments of Matsunaga and Simpson (240), using allophenic and bone marrow radiation chimeras, can be interpreted to suggest an Ia-restricted T*h*-CTLp interaction, although other explanations can be made for these observations (29). In these studies it is unclear if the observed H-2 restriction occurs at the level of antigen recognition or at the level of collaboration between T*h* and CTLp.

The experiments of Plate and her colleagues (202,241) indicate that soluble T*h* derived factors exhibit MHC-restriction in the triggering of CTLp. This restriction is obscured by the presence of Ia$^+$ T cells in culture. In the absence of Ia$^+$ T cells, MHC-restricted triggering by these factors involves mainly the responses of Lyt-1,2,3$^+$ CTLp to haptenated-self molecules but not to MHC alloantigens. This suggests that more immature CTLp that have not previously received inductive stimuli due to environmental priming may be required to observe MHC-restricted interactions between T*h* and CTLp. However, in these studies it was not determined at which level this restriction occurs.

Although CTLp can be activated in the absence of Ia$^+$ APC, it is unclear if all CTLp lack this restriction. The demonstration that activation of a H-2D$^d$ -reactive CTL hybridoma is facilitated by Ia$^+$ stimulators suggests that other CTL may require interactions with Ia molecules (188). In addition to or instead of anchoring T*h* and CTLp together for the delivery of antigen nonspecific inductive signals, endogenously-expressed or passively acquired MHC molecules on T*h* or APC may function to interact with molecules in a CTR complex for the delivery of a functionally complete inductive signal.

The requirement for close spatial proximity of the determinants recognized by T*h* and the precursors of AFC or DTH effector cells is well established (10,15,16,18-20,25,27,28). In contrast, CTL can be triggered by T*h* recognizing antigens that are physically separated from those recognized by CTLp (127,197,242,243). Furthermore, antigen-nonspecific and MHC-unrestricted lymphokines can also trigger CTLp in the absence of T*h* cells (52,54,244). On the surface, this suggests that CTLp have less stringent triggering requirements than AFC, DTH, or the T*h* that participate in DTH induction. However, a number of experiments indicate an important role for linked recognition in the induction of CTLp.

The experiments of Sopori *et al.* suggest that linkage of determinants recognized by T*h* and CTLp is required for thymocyte- but not for splenic-CTLp (245). The CTL responses of AQR splenocytes to the H-2K$^k$ antigens of B10.A mice was augmented in a dose-dependent manner by the addition of B10.T(6R) stimulator cells, which differ from AQR in the I through S regions of H-2. In contrast, thymocyte CTLp were not induced using this protocol in which the helper- and CTL-recognized determinants were on physically separate cells. These thymocyte CTLp and/or T*h* populations appear to be less susceptible to "unlinked" triggering. It has been demonstrated that thymocytes that bind peanut agglutinin (PNA) are unresponsive to alloantigen either in the presence or absence of T cell growth factors, including IL2 (246-248). However, it is unclear if this supposedly "dead-end" population may in fact be triggered only by antigen-specific events requiring linkage of determinants recognized by T*h* and CTLp.

The studies of Keene and Forman (249) demonstrate a requirement for linkage of determinants recognized by CTLp and T*h* for *in vivo* priming of CTL responses to Qa-1 antigens. Cytotoxic responses against Qa-1 were generated only if female mice were primed with cells expressing both Qa-1 alloantigens and male histocompatibility antigens (HY), but not if they were primed with syngeneic male cells plus female cells expressing Qa-1 alloantigens. Similar studies by Kanagawa *et al.* (250) demonstrate a requirement for the expression of foreign minor histocompatibility antigens (mH) and HY on the same immunizing cells in order to elicit CTL responses against HY antigens in female CBA mice. Other studies also suggest that linkage of determinants recognized by CTLp and T*h* more effectively stimulates CTLp than does antigen in an unlinked form (251, N.Gascoigne, personal communication). All members of the CTLp population may not be equally susceptible to triggering by antigen-nonspecific and MHC-unrestricted inductive signals such as IL2.

Since the initial demonstration that soluble factors from antigen-stimulated lymphocytes were able to substitute for intact T*h* in the induction of CTLp (244), much effort has been expended in the biochemical and functional characterization of these soluble antigen-nonspecific factors. In the mouse, antigen- or mitogen-activated Lyt-$1^+$ T cells release IL2 which then participates in the triggering of CTLp that have expressed IL2 receptors after antigen recognition (52,54,203,252-256). Unlike these CTL that require exogenously provided

IL2 and perhaps other lymphokines, CTL clones have been reported that are able to produce sufficient IL2 to induce their own proliferation after stimulation by alloantigen-expressing stimulator cells or Sepharose-linked antibodies recognizing molecules in the CTR complex (144,188,189,222). The physiological significange of these observed differences in requirements for exogenously-provided IL2 are unclear. IL2 appears to stimulate CTLp proliferation after recognition of antigen (256-258). It is unclear if IL2 also stimulates the production of other factors involved in the triggering of CTL responses.

Antigen-nonspecific lymphokines in addition to IL2 have been reported to participate in the induction of CTLp (259-268). In contrast to IL2 which stimulates CTLp proliferation, these additional factor(s) stimulate differentiation of CTLp into CTL that are functionally capable of delivering a lytic signal to targets expressing determinants recognized by their clonally-expressed CTR (260,261). Biochemical criteria are also able to distinguish these CTL differentiation factors (CTDF) from IL2, IL1, or $\gamma$-interferon (258,260,263,264,266-268). Recent evidence demonstrates that IL3 is at least one of the CTDF that have been reported (267). Functional CTDF activity has been identified in supernatants from T cell lines and hybridomas, EL4 thymoma cells, MØ-like spleen cells and MØ-like tumor cells (WEHI-3), in addition to supernatants from mitogen- or antigen-activated spleen cells (257-268). Production of IL3 by MØ may explain the significant enhancement of cloned alloreactive CTL by adherent cells (143). It is not known if production of IL3 (CTDF) by dendritic cells is also able to enhance CTL activity. In addition to producing CTDF (IL3) for CTLp induction, both T cells and MØ may be targets for its activity as well (56,269). The recent cloning of the murine IL3 genes (270) will facilitate the analysis of its role in the induction of CTLp.

It is clear that some CTLp require at least three signals for induction - antigen, IL2, and CTDF (IL3). However, evidence presented in this thesis and elsewhere (29,202,239,271-273) demonstrates that antigen-specific T*h*-derived inductive signals are able to participate in the induction of CTLp. The number and type of signals a CTLp has previously received will determine its requirments for additional inductive signals. In the absence of inhibitory activity, antigen-nonspecific lymphokines that are active *in vitro* may be able to bypass requirements for functionally relevant clustering of molecules in a CTR complex. Antigen-nonspecific lymphokines, namely IL2 and CTDF may constitute additional signals that mediate clonal expansion and differentiation, respectively.

## F. The Role of Soluble Helper T Lymphocyte Receptors in the Induction of Cell-Mediated and Antibody-Mediated Responses

In 1968 Bretscher and Cohn proposed an antigen-specific second signal for the selective induction of lymphocytes that react with non-self antigens (30). Selective pressures that prevent the induction of self-reactive T*h* would minimize the induction of autoimmune responses by T*h*-derived ASHF. The soluble nature of ASHF would enable T*h* to extend their ability to deliver antigen-specific help beyond the limits of the T*h* cell membrane.

Recent genetic and biochemical analysis has revealed similarities between T*h*AR and immunoglobulins (43-46,147,148,151-154), possibly due to duplication of primordial genes. The precedent of distinct membrane and secreted forms of $\mu$, $\delta$, and $\gamma$ immunoglobulin heavy chains suggests that T*h*AR may have used similar recombinatorial mechanisms to generate secreted and membrane-bound forms of its receptors (155-157,221,274-277). Distinct secreted and membrane-bound forms of IgM are present in species as phylogenetically ancient as sharks (278).

The first experimental evidence in support of a soluble, antigen-specific, T cell-derived signal was provided in 1972 by Feldman and Basten's analysis of antibody responses (279). They demonstrated antigen-specific, T cell-derived inductive signals that acted on B lymphocytes and/or MØ separated from the T*h* by a cell-impermeable ($0.1\mu m$ pore size) membrane. These initial observations of B cell induction by antigen-specific T*h*-derived products, presumably T*h*AR, have been confirmed in a wide-variety of human and murine experimental systems anlyzing both primary and secondary antibody responses (reviewed in 17,280-282). Tada and others (17,50,51,280,283-285) have reported a similar antigen-specific

T*h*-derived factor that helps secondary antibody responses but differs from the type of ASHF reported by Feldman (279) in its action on T cells rather than on B cells or MØ.

An apparent heresy against conventional dogma (159,160) is that soluble T*h*AR, unlike most but apparently not all of their membrane-bound counterparts (160-164), are able to bind nominal antigens in the apparent absence of MHC products. Affinity columns consisting of Sepharose-coupled antigens such as CRBC, synthetic polypeptides, serum proteins, ovalbumin (OVA), or haptens, have been demonstrated to bind functionally active helper molecule in a specific manner (239,286-294).

Other interpretations of this apparent lack of H-2 restriction in recognition of nominal antigen can be made to suggest that the binding of ASHF to nominal antigen still has an H-2-restricted component: 1. the affinity of binding by ASHF to nominal antigen could conceivably be greatly increased when nominal antigen is presented in the context of MHC products; 2. conformational determinants on nominal antigens reocgnized by ASHF may resemble an antigenic determinant ($X$) presented in the context of self MHC molecules (nominal antigen $\cong$ Self$+X$); 3. ASHF moities that contain MHC-encoded molecules may provide their own elements for MHC-restricted recognition of antigen and thus appear unrestricted. In spite of these caveats, based on the binding of ASHF to antigen-affinity columns in the absence of H-2-encoded products and the differences in the requirements for H-2-encoded products of $Ca^{++}$-sufficient and $Ca^{++}$-deficient ASHF (29,239, and Chapter III of this thesis), it is suggested that ASHF binding of antigen can be essentially MHC-unrestricted. However, MHC-encoded molecules derived from adherent cells may be required at other steps of CTLp induction that do not involve recognition of nominal antigen.

Although most ASHF preparations are able to bind nominal antigens in the absence of MHC products, some reports suggest Ia-restricted interactions of ASHF in the induction of antibody responses. It has been observed that B lymphocytes and ASHF-producing T*h* must be syngeneic in at least the IA-subregion of the MHC in order for induction to occur (50,290,295,296). In contrast, other reports have demonstrated that induction of B cells by

ASHF is not restricted by a requirement for identity in any region of H-2 between ASHF-producing cells and B lymphocytes (287,297,298).

Analysis of antibody responses to synthetic polypeptides such as Poly-L-(tyrosine, glutamic acid)-poly-DL-alanine-poly-L-lysine (TGAL) that are controlled by I-region genes has been used to define the roles of these genes in the production of ASHF and in the ability of B cells to bind and be triggered by ASHF (282,297,298). By analysis of the abilities of TGAL-low-responder mice to produce ASHF or to be triggered by ASHF, it was concluded that three types of defects contribute to the low-responder status of some mice: 1. some strains of low-responder mice are unable to produce ASHF in response to TGAL but are able to respond to ASHF; 2. some low-responder mice are able to produce ASHF but are unable to respond to it; and 3. some mice exhibit defects at both levels 1 and 2.

Gene complementation was suggested by the ability of F1 hybrids between low-responder mice that express a defect at level 1 (B10.M or A.SW) and those expressing a defect at level 2 (B10.BR or I/St) to respond to TGAL (282,298). However, this complementation was not able to be consistently reproduced even by Taussig and his colleagues (282,299-301).

It was observed that non-MHC-linked genes, possibly immunoglobulin heavy chain-linked genes, contribute to both the ability to produce TGAL-specific ASHF and the ability to respond to it (282,302). To explain these observations a minimal four gene factor/acceptor model was proposed (282). In this model two different MHC-unlinked genes are involved in the MHC-linked expression of ASHF or its acceptor. Further experimental evidence in support of this model, however, is lacking. Genetic and biochemical analysis of Ia determinants on ASHF and their complementary acceptor molecules on B cells will be required to resolve the phenomenological conflicts involving the roles of MHC- and non-MHC products in ASHF-mediated lymphocyte induction.

The mechanisms of ASHF-mediated induction of B lymphocytes are not clear. Some reports demonstrate that at least one type of ASHF mediates its effects on B cells indirectly via interactions with T cells (17,50,51). In contrast, other reports of the ability of ASHF to trigger AFC responses from T-depleted spleen cells indicates that a different type of ASHF acts directly on B cells or indirectly via MØ-like cells (279,287,294,295,303,304). Antibody responses have also been induced *in vivo* by ASHF in nude mice (305).

It has been demonstrated that bone marrow cells (298) or purified B cells (294) can bind ASHF in the absence of antigen although antigen is required for induction. In the studies of Shiozawa *et al.* (294) the "B cell" population contained less than 0.1% MØ, as determined by the uptake of polystyrene beads. This, however, does not eliminate the possible contribution of dendritic cells. It does suggest that ASHF can act directly on B cells but it is not known if ASHF can also be absorbed by MØ and/or dendritic cells.

Diener *et al.* have proposed that ASHF is an amphipathic molecule that exerts its effects upon insertion into the the phospholipid bilayer of the B lymphocyte membrane (306). However, ASHF could lack hydrophobic segments but be attached to the cell surface by ASHF "acceptors", as suggested by Munro and Taussig (301). The model of Diener suggests that the positioning of ASHF molecules among the immunoglobulin receptors on B cells may facilitate antigen-mediated clustering of immunoglobulin receptors for the delivery of a functionally complete inductive signal. This model is consistent with the requirement for close spatial proximity of antigenic determinants recognized by T*h* and B cells (15-20). Recently, Dekruyff *et al.* (291) have demonstrated a requirement for linked recognition in the ASHF-mediated induction of antibody responses although earlier reports have demonstrated ASHF-induced triggering of "bystander" lymphocytes in an unlinked manner (307,308).

Two basic types of geometric configurations can be suggested for ASHF-mediated clustering of immunglobulin receptors on B cells: 1. T*h*AR may be positioned among immunoglobulin molecules with their tails in or on the cell-membrane and their antigen recognition units exposed to the universe of self and non-self. 2. An alternative configuration would have the antigen-binding cleft of ASHF facing the immunoglobulin (or T cell receptor) antigen-binding cleft across an antigen bridge. Either or both of these mechanisms may be operating in ASHF-mediated induction of B and T lymphocytes.

Experiments by Jaworski *et al.* (290) dissected the induction of B lymphocytes into proliferative and differentiative signals. The former signal, provided by antigen and ASHF, required at least partial H-2 compatibility between the ASHF-producing cells and B lymphocytes. The stimuli for differentiation into AFC were soluble, antigen-nonspecific, H-2-unrestricted signals derived from T*h* and M0-like cells. Many laboratories are currently investigating the molecular and functional characteristics of these nonspecific factors that participate in the stimulation of antibody secretion.

A number of reports suggest that ASHF are a complex of T*h*AR and Ia antigens. Molecular weight estimates range from 40 to 70 Kd (280-282,309). However, precise biochemical characterization of ASHF subunits has not been achieved. The antigen-specifity of ASHF in terms of binding and functional activity strongly suggests but does not prove that ASHF are the clonally-expressed antigen-receptors of T*h*. Antisera raised against whole mouse immunoglobulin does not react with ASHF but Sepharose-coupled antibodies recognizing framework determinants of the immunoglobulin heavy chain variable region (Vh) have been able to deplete ASHF activity (288,298,310,311). In light of the recently reported homolgies between immunoglobulins and T*h*AR $\alpha$ and $\beta$ chains (44,45) cross-reactivities between these types of molecules are not really surprising. However, depletion of ASHF activity by immunoadsorption with monoclonal antibodies known to recognize V or C region determinants of T*h*AR is needed to conclusively demonstrate that ASHF contain T*h*AR $\alpha$ and/or $\beta$ chains.

Many laboratories have independently reported depletion of functional ASHF activity by conventional or monoclonal antibodies recognizing Ia determinants (30,271,287,290,311-313). The studies of Lonai *et al.* demonstrate that determinants expressed on at least I-A$\beta$ and I-E$\alpha$ are expressed on ASHF (296). It is interesting that although this ASHF is IA$\beta^+$, it requires identity in the K-IA regions with B cells and M0 in order to trigger antibody responses. This could reflect like-like interactions between polymorphic IA$\beta$ determinants on the ASHF and B lymphocytes or M0. Alternatively, it may be due to a requirement for interactions of polymorphic IA$\beta$ or IE$\alpha$ determinants on ASHF with IA$\alpha$ or IE$\beta$ determinants, respectively, on B cells, MØ, or even with those molecules which have been passively-absorbed onto T lymphocytes. It is possible that Ia determinants unique to T cells (314,315) may be found on ASHF or may be acceptor molecules for determinants expressed on ASHF moities. Other non-MHC-encoded, L3T4-like molecules (186-188) may be involved in the binding of soluble T*h*AR-Ia complexes to B cells, MØ, or T cells.

It is suggested in this thesis that Ia antigens constitue a polymorphic glue that binds T*h*AR together. Complexes of Ia,antigen, and T*h*AR may be released from T*h* after activation for the delivery of inductive signals to lymphocytes recognizing determinants that are physically linked to the determinants recognized by clonally-expressed T*h*AR. Quantitative and/or qualitative variations of nonclonally-expressed interaction structures on ASHF or on the precursors of cellular effectors or AFC may dictate the ability of an ASHF moiety to trigger T cell or B cell subsets.

As conventional Ia determinants found on ASHF are not constitutively-expressed on murine T lymphocytes, these Ia molecules on ASHF are probably derived from adherent cells, as our own work suggests. Thus the subunits of ASHF appear to be derived from two distinct cell types and assembled extracellularly. This is different from the assembly of immunoglobulins in which both heavy and light chain subunits are produced by the same B cell. However, by analogy to the proposed model of ASHF, the antigen-specific suppressor factors characterized in the laboratory of the late R.K. Gershon consist of an antigen-binding subunit produced by Lyt$1^+2^-$ cells and a "carrier" subunit produced by Lyt$1^-2^+$ cells (316,317). In this model I-J products also appear to function as a polymorphic glue. Other models of antigen-specific suppressor factors bear resemblance to the proposed ASHF model in that both H-2-encoded and non-H-2-encoded subunits appear to be required for functional activity (318,319). The maintenance of related biological functions on separate polypeptide chains or at least encoded by separate exons may permit greater adaptability to environmental selective pressures.

Although the effects of ASHF have been studied most extensively in the induction of B cell responses, ASHF have also been reported to participate in the induction of CTL and DTH responses. Kilburn *et al.* (271) provided the first evidence for a factor that specifically enhanced a CTL response to a syngeneic tumor (P815). Like many of the other reports, this ASHF bound to antigen-affinity columns, and anti-Ia or anti-H-2 columns but not to an anti-mouse immunoglobulin column. Its molecular weight was estimated to be 50-66 Kd and analysis of its functional activity distinguished it from IL2 (272). Dr. Kilburn and his colleagues have independently reproduced the ASHF-mediated induction of thymocyte CTLp, using the system developed by Dr. Pilarski and myself (29,239), in the criss-cross specificity analysis of KLH- and PPD-reactive ASHF (D. Kilburn, personal communication). Recently they have also developed a radioimmune assay to quantitate ASHF by its ability to block binding of free antibody to immobilized antigen (320).

Experiments by Plate *et al.* (202) demonstrated that antigen-specific helper activity for CTL responses were present in 6-hour supernatants of B10.A(18R) stimulator cells cultured with draining lymph node cells from C57Bl/10 mice that had previously received a body or tail skin graft from B10.A(18R) mice. Using a panel of glutaraldehyde-fixed stimulator cells from congenic mice, the antigen-specificity of this soluble helper activity was mapped to H-2D$^d$ (on B10.A(18R) stimulator cells). This ASHF activity was detectable in supernatants from lymph node cells harvested as early as four days after skin grafting. In contrast, IL2 activity was not detectable in supernatants from draining lymph nodes harvested until approximately 10 days after skin grafting. It was not determined if the ASHF-producing cells required identity in any region of H-2 with CTLp, T*h*, or adherent cells in the responder population in order to induce CTL responses.

ASHF-activity for thymic CTLp was detected in supernatants from spleen and lymph node cells of cyclophosphamide-treated, trinitrochlorobenzene-painted mice that were cultured with haptenated syngeneic spleen cells (273). Production of ASHF in this system required stimulation of primed Thyl$^+$,Lytl$^+$2$^-$ cells with specific antigen. Nonspecific lymphokines that acted on alloreactive CTLp were present in supernatants collected 5 hours after stimulation. In contrast, ASHF predominated in supernatants collected 25 hours after stimulation. The molecular weight of this ASHF was estimated to be approximately 43 Kd, as determined by gel filtration analysis. This molecular weight is consistent with those previously reported for murine $\alpha$ and $\beta$ chains of T lymphocyte antigen receptors (42-46).

Studies by Colizzi *et al.* (293) provide the first evidence of the antigen-specific induction of DTH responses by soluble factors. Their ASHF is present in the 24-hour supernatants of draining lymph node cells harvested 4 days after skin painting with contact sensitizing agents. ASHF-activity was able to bind specifically to hapten-Sepharose columns in the apparent absence of MHC-products. Picrylated or oxazolonated spleen cells were treated with affinity-purified ASHF prior to inoculation into foot pads. Five days later, the sensitizing agent or the control hapten was applied and ear-swelling was measured 24 hours later. Hapten-specific helper activity was produced by $Lyl^{+}2^{-}$, $IA^{+}$, $IJ^{-}$ T cells. Presumably, the IA on these T cells was passively acquired from MØ-like cells. These 50-70 Kd, soluble ASHF also contained determinants reactive with monoclonal anti-IA antibodies.

The relationships among the ASHF characterized by a wide variety of experimental systems are unclear. Can one type of ASHF deliver an inductive signal to both B and T cells? Previously, systems have not been developed to answer this question. Do the subunits of ASHF define the target cells with which it preferentially interacts? What are the roles of interactions between clonally-expressed antigen-binding units and nonclonally-expressed acceptor sites with MHC-encoded molecules? These questions will be dealt with in this thesis. The following general model of lymphocyte induction by ASHF is proposed:

1. ASHF induce immune responses by clustering or otherwise inducing conformational changes in functionally related clonally-expressed and nonclonally-expressed molecules on lymphocyte membranes.

2. Lymphocyte activation involves H-2-encoded molecules and close physical proximity of determinants recognized by ASHF and the precursors of effector or regulatory lymphocytes. These requirements may be obscured by the effects of antigen-nonspecific lymphokines.

3. MHC-encoded subunits of ASHF function as a polymorphic glue to attach T*h*AR to each other and to the precursors of effector or regulatory cells for interaction with lymphocyte receptors to deliver an inductive signal. Additionally, they may determine the type of lymphocyte with which a given form of ASHF is able to interact.

4. Antigen nonspecific lymphokines mediate maturation and clonal expansion, secondary to the effects of ASHF.

As a student it was not possible to test all aspects of this model. However, a unique system was developed to demonstrate a role for ASHF in the induction of CTLp and to answer some of these questions. Evidence in support of this model is presented in this thesis.

# II. MATERIALS AND METHODS

This chapter describes the basic procedures used in experiments reported in this thesis and elsewhere (29,239). Other methods are described in the appropriate chapters.

*Animals.* BALB/cCr, C57Bl/10J (B10), CBA/J, B10.BR and (BALB/cCr x CBA/J)F1 mice were bred at Ellerslie Animal Farm, University of Alberta. B10.A(5R) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice used were 4 to 16 weeks old and were sex-matched in individual experiments.

*Antigens.* Erythrocytes were obtained from chickens homozygous for the *B*2, *B*13, or *B*19 MHC haplotypes. These chickens were bred by the Biosciences Animal Services, University of Alberta. Burro red blood cells (BRBC) were obtained from the Colorado Serum Company (Denver, CO). Freeze-thaw antigen (FTA) was prepared as described previously (215). Briefly, washed erythrocytes were suspended in Eagle's Minimal Essential Medium (MEM) (GIBCO - Burlington, Ontario) containing $5 \times 10^{-5}$M $\beta$-mercaptoethanol (Eastman Kodak - Rochester, NY) at a concentration of $10^{8}$ or $10^{9}$ per ml. Subsequently, the erythrocytes were lysed by four cycles of freezing in liquid $N_2$ and thawing at 37°C. The lysate was centrifuged at 500 x G for 5 minutes to remove any residual cells and cellular debris. The supernatant (FTA) was stored at -70°C or in liquid $N_2$ until use.

Keyhole limpet hemocyanin (KLH), purchased from Calbiochem (La Jolla, CA), was provided by Dr. E. Diener. *Corynebacterium parvum (CP)* was prepared by Dr. K.C. Lee, as described previously (323). Purified protein derivative (PPD) of tuberculin (Connaught Laboratories - Willowdale, Ontario) was also provided by Dr. K.C. Lee.

*Preparation and Purification of IL2.* Interleukin 2 was partially-purified from supernatants of phorbol-12 -myristate-13-acetate (PMA)-stimulated EL4.E1 thymoma cells (321), using procedures developed in the laboratory of Dr. V. Paetkau, as described previously (52,254). Irradiated (500 rad) C57Bl10/J mice were inoculated intraperitoneally with $3 \times 10^{6}$ El4.E1 thymoma cells. Approximately two weeks later, ascites was harvested.

Washed cells were suspended in RPMI 1640 medium (GIBCO - Burlington, Ontario) containing 4% horse serum (GIBCO), $5 \times 10^{-5}$M $\beta$-mercaptoethanol (Eastman Kodak - Rochester, NY) and 10ng/ml of PMA (Sigma - St. Louis, MO). After 18-24 hrs incubation at 37°C in a 10% $CO_2$-air atmosphere, the culture fluid was centrifuged at 500 x G to remove cells and debris.

Ammonium sulfate (Fisher Scientific - Edmonton, Alberta) was added to 80% saturation (560g/L) and stirred at 4°C overnight. Subsequently, the precipitate was centrifuged at 3400 x G for 1.5 hours. The supernatant was discarded and the pellet suspended in a minimal volume of double-distilled $H_2O$. This was dialyzed exhaustively against 0.01 M HEPES/0.05 M NaCl (pH 7.4). This material (Fraction II) was purified further on a Sephadex G-100 (Pharmacia - Uppsala,Sweden) gel filtration column. Fractions containing $\geqq$30% of optimal IL2 activity were pooled, concentrated, and filtered through 0.2 $\mu$m membranes, and stored at -70°C. For convenience this G-100-purified material (Fraction III) will be referred to as IL2 throughout this thesis, although Fraction III is known to contain a variety of other antigen-nonspecific lymphokines (V. Paetkau, personal communication). SDS-PAGE electrophoresis also demonstrates that Fraction III is a very heterogeneous mixture of proteins (J.K., unpublished observations).

*Helper T Cell Lines.* The T cell line CHI that recognizes *B*2-CRBC antigens was prepared by Dr. C. Shiozawa, as described previously (288). Briefly, CBA/J thymocytes were "educated" with CRBC in irradiated syngeneic recipients. After 7 days spleens were harvested and the recovered cells (about 15% of the $1.5 \times 10^8$ intravenously inoculated thymocytes) were cultured in the laboratory of Dr. V. Paetkau. Initially cells were cultured with antigen, irradiated syngeneic feeder cells and IL2 in RPMI 1640 medium containing 50 $\mu$M $\beta$-mercaptoethanol, 10% fetal calf serum (FCS), and antibiotics. Clone 4C6 was derived from the parent line CHI by seeding cells at a concentration of 0.2 cells per microculture well containing an optimal concentration of IL2 and irradiated (2500 rad) feeder cells. For maintenance of T*h* cells and preparation of ASHF, T*h* lines are cultured in the absence of antigen and feeder cells.

The T*h* line L61 that recognizes *B*19 CRBC antigens was developed by Drs. T. Mosmann (DNAX Research Institute - Palo Alto, CA) and C. Shiozawa using methods similar to those used for the development of CHI. The T*h* lines CPPD and D*CP* that proliferate specifically in response to PPD and *Corynebacterium parvum*, respectively were developed by C. Guidos in the laboratory of Dr. K.C. Lee by the methods of Kimoto and Fathman (322) as described elsewhere (323). The CPPD and the D*CP* lines are of CBA/CaJ and DBA/2J origin, respectively. The CKA T*h* line that recognizes KLH was developed by Dr. A. Sinha from CBA/CaJ mice by the methods of Kimoto and Fathman (322).

*Preparation of Antigen-Specific Helper Factors.* Supernatants from the T*h* line CHI were collected after 24-48 hours of culture and dialyzed against phosphate buffered saline (PBS). These supernatants were passed through antigen-affinity columns that consisted of *B*2- or *B*13-CRBC coupled to Sepharose 4B as described previously (290). The bound material was eluted from the CRBC with 2M NaCl, dialyzed against PBS, and concentrated. This material is referred to as $Ca^{++}$-sufficient ASHF. $Ca^{++}$-deficient, i.e. "incomplete" ASHF was prepared by dialyzing the $Ca^{++}$-sufficient ASHF against 0.01 M HEPES buffer (pH 7.4) containing $10^{-3}$M Ethylene Glycol-bis-($\beta$-Aminoethyl ether) N,N'-Tetra-acetic Acid (EGTA). This material was re-chromatographed on a *B*2-CRBC-affinity column in the presence of $10^{-3}$ M EGTA. The bound material was eluted from the antigen-affinity column with 2M NaCl, exhaustively dialyzed against PBS, and concentrated. Protein concentrations were determined by absorbancy at 280 nm using bovine serum albumin as a standard.

*Assay for Antigen-Specific Help for CTLp.* Three x $10^5$ mouse spleen cells in MEM were incubated in the presence or absence of "carrier" antigens recognized by T*h* or ASHF at 37°C for 2 hours in V-bottom microtiter wells (Linbro-Hamden, CT). In the experiments using erythrocyte FTA, the equivalent of $10^6$ RBC were added to each well. Unbound antigen and non-adherent cells were then removed by gently washing the cultures twice with MEM. Graded dilutions of T*h*, ASHF, or an optimal concentration of partially-purified IL2 were prepared in

MEM containing 4% gammaglobulin-free horse serum and 1% FCS (GIBCO) or 5% FCS, 5 x $10^{-5}$M $\beta$-mercaptoethanol and antibiotics, and added to the adherent stimulator cells. The quantity of IL2 (Fraction III) required to induce optimal CTL responses in each microwell was derived from approximately 600 to 1200 PMA-stimulated EL4.E1 cells. Cultures were irradiated with 1500 rads prior to the addition of 1 or 3 x $10^5$ thymocytes from 4-6 week-old mice per culture. Cultures were incubated at 37°C in a 10% $CO_2$-air atmosphere for 5 days.

*Assay for IL2 Activity.* Antigen-specific helper factor preparations and mixed lymphokine preparations from PMA-stimulated cultures of EL4.E1 cells were assayed for their ability to support the survival of an IL2-dependent alloreactive CTL line MTL 2.8.2 (324). Survival of the indicator cells was assayed using the 3-(4,5-Dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma - St.Louis, MO) system developed by Dr. T. Mosmann (DNAX - Palo Alto, CA) as described recently (325). Graded dilutions of samples to be tested for IL2 activity were added to flat-bottom microtiter wells (Linbro - Hamden, CT) containing 2 x $10^4$ indicator cells. After a 24 hr. incubation at 37°C, 0.01 ml MTT (5 mg/ml in PBS) was added to each microtiter well. Cultures were reincubated at 37°C for four hours to permit cleavage of MTT. To solubilize any of the blue formazan cleavage product of MTT that had formed during the four hour incubation, 0.1 ml of 0.04 N HCl in isopropanol was added to each well and vigorously mixed. Optical density (O.D.) was measured at 595 nm. This MTT assay system is comparable in accuracy and sensitivity to radionucleotide incorporation for the quantitation of cellular survival and proliferation (325 and unpublished observations), but it is much quicker to perform and does not require the handling of radioactive material.

*Preparation of Blast Target Cells.* Spleen cells were harvested in Leibowitz medium (GIBCO) containing 0.1% gelatin (MCB - Norwood, Ohio). One x $10^7$ cells were cultured in Costar (Cambridge, Mass.) wells containing 5 ml of media (MEM with 5% FCS, 50 $\mu$M $\beta$-mercaptoethanol and 8$\mu$g Concanavalin A (Con A) (Calbiochem - La Jolla, CA). Cells were cultured for 2 days at 37°C in a 10% $CO_2$ atmosphere prior to their use as target cells in a cytotoxicity assay. Target cells in all cytotoxicity assays were not pulsed with any carrier antigens.

*Cytotoxicity Assay.* Cytotoxicity generated against the murine MHC alloantigens on the stimulator cells was asayed using Con A-induced blast target cells in a standard $^{51}$Chromium release assay which has been described previously (126). Six test wells per group were assayed individually using 5 x $10^4$ target cells per well. Spontaneous/ detergent release of $^{51}$Cr from blast target cells was generally between 0.2 and 0.35. Cytotoxicity is expressed as % Specific Lysis±S.E.. Killer to target ratios were in the order of 0.1-1:1.

*Antiserum Treatment of Cell Suspensions* Cells were suspended at $10^6$/0.5 ml in Leibowitz medium (GIBCO - Burlington, Ontario) containing 0.1% gelatin (MCB - Norwood, Ohio) and an optimal dilution of monclonal antibodies or conventional antisera. These anti-Lyt 1, Lyt 2, Thy 1, and $Ia^k$ (10-3.6 - anti-IA$\beta$)antibodies have been characterized previously for their ability to deplete functional cell types (326,327). After incubation on ice for one hour the cells were pelleted and resuspended in medium containing an optimal dilution of self-prepared rabbit complement (C'). After incubation for 45 minutes at 37°C, cells were washed and counted. Cell viability was determined by eosin dye exclusion (328). Lysis of cells by C' alone was less than 4%.

# III. INDUCTION OF CYTOTOXIC LYMPHOCYTE RESPONSES BY ANTIGEN-SPECIFIC HELPER FACTORS

## A. Introduction

Many reports document the ability of soluble antigen-specific "factors" to induce cell-mediated of antibody-mediated immune responses, as reviewed previously in this thesis. The following three chapters describe characterization of ASHF participating in the induction of CTLp.

Research over the past decade by Drs. Diener, Shiozawa, Singh, and their colleagues in Edmonton has characterized the role of ASHF in the induction of primary AFC responses to a wide variety of antigens, including CRBC (290,294,295,304,306,313). Our initial objective was to determine if ASHF, derived from T*h* lines and clones that specifically recognize CRBC antigens, are able to participate in the induction of CTLp. In order to facilitate our analysis we have separated ASHF from non-specific lymphokines by antigen-affinity chromatography.

Because CRBC antigens, recognized by our ASHF, are not constitutively present on mouse stimulator or target cells used in a CTL assay , it was necessary to redesign our basic assay system for helper function. We have modified the helper-dependent thymocyte CTLp induction assay, developed by Pilarski (125), to test the ability of ASHF to function as a helper molecule in CTLp induction. In this report we describe an *in vitro* system, analogous to classical "hapten-carrier" models, for the analysis of the role of ASHF in the induction of CTLp.

The collaborative interactions among CTLp, ASHF (or T*h*), and APC are illustrated in Figure 1. In order to introduce CRBC determinants onto the surface of mouse stimulator cells, CRBC fragments are fed to adherent spleen cells to provide "carrier" determinants recognized by T*h* cells or ASHF. Recognition of the appropriate carrier determinants by T*h* or ASHF enables thymocyte CTLp to respond to H-2-encoded stimulator cell alloantigens that are operationally defined as the "hapten" determinants. Although murine MHC antigens are not

Figure 1

Collaboration in ASHF-mediated induction of CTLp

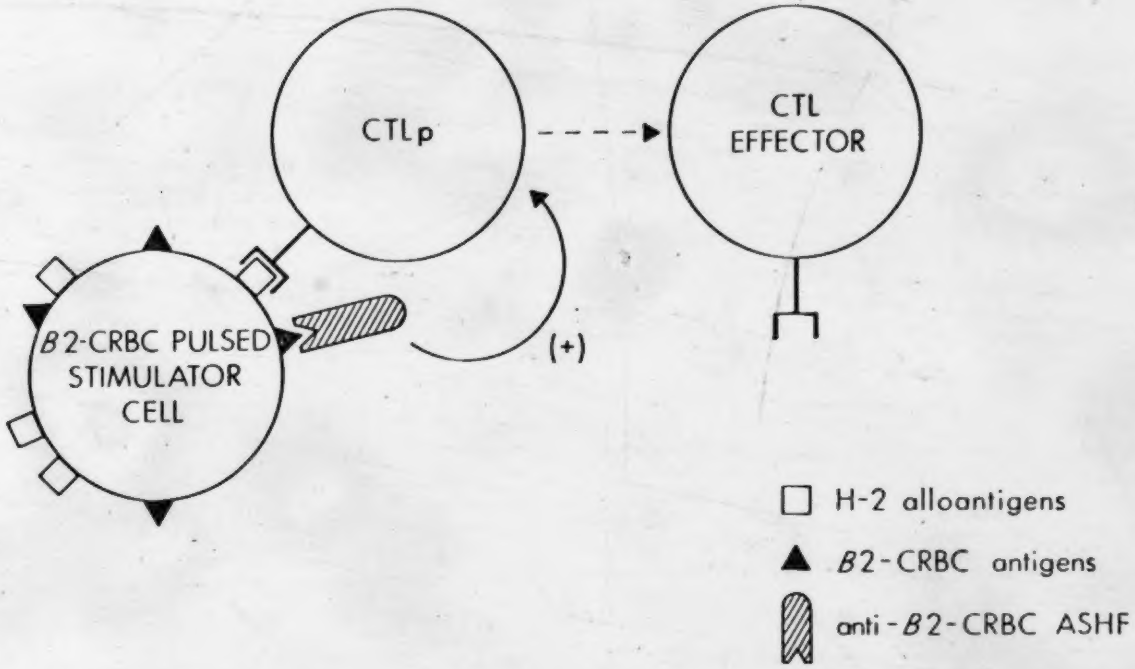

CTLp
CTL EFFECTOR
B2-CRBC PULSED STIMULATOR CELL
(+)
H-2 alloantigens
B2-CRBC antigens
anti-B2-CRBC ASHF

Figure 1: For reasons of simplicity, the CTLp is presented as the recipient of antigen-specific help (+). The model above may also apply to precursors of T*h* that, once triggered by ASHF (or T*h*), induce CTLp.

conventional "haptens", this terminology is used to distinguish between the determinants recognized by the T*h* or ASHF (carrier-specific) and the CTLp (hapten-specific) in this system. Precedent exists in the literature for the operational definition of macromolecular structures as haptens (10,329).

The irradiated adherent stimulator cells have at least two functions in this system. They constitutively express H-2 alloantigens recognized by the CTLp and they also "present" exogenously added "carrier" determinants recognized by T*h* or ASHF. The system developed here is basically analogous to induction systems in which T*h* recognize Class II MHC antigens and the CTLp recognize Class I MHC antigens (110,111).

Previous studies in the induction of B cells by ASHF have indicated that $Ca^{++}$ is required to maintain the functional association of antigen-specific and antigen-nonspecific subunits of ASHF (294). To determine if the ASHF that participates in the induction of CTLp is also composed of distinct subunits requiring $Ca^{++}$ for their association, the antigen-specificity and H-2 restriction properties of ASHF, purified by antigen-affinity chromatography in the presence or absence of $Ca^{++}$, were examined.

## B. Materials and Methods

The materials and methods used in this chapter are all described in Chapter II. All ASHF used in experiments presented in this chapter were prepared by Dr.. C. Shiozawa. The basic experimental design is illustrated in Figure 2.

## C. Results

### Helper T Cell Lines Induce CTL Responses in a Carrier-Specific Manner

To determine the potential of our "hapten-carrier" system for the analysis of CTL induction from thymocyte precursors, our initial experiments utilized T*h* lines. These T*h* recognize polymorphic CRBC antigens that are encoded by the chicken MHC (*B* locus). We

Figure 2

Experimental design

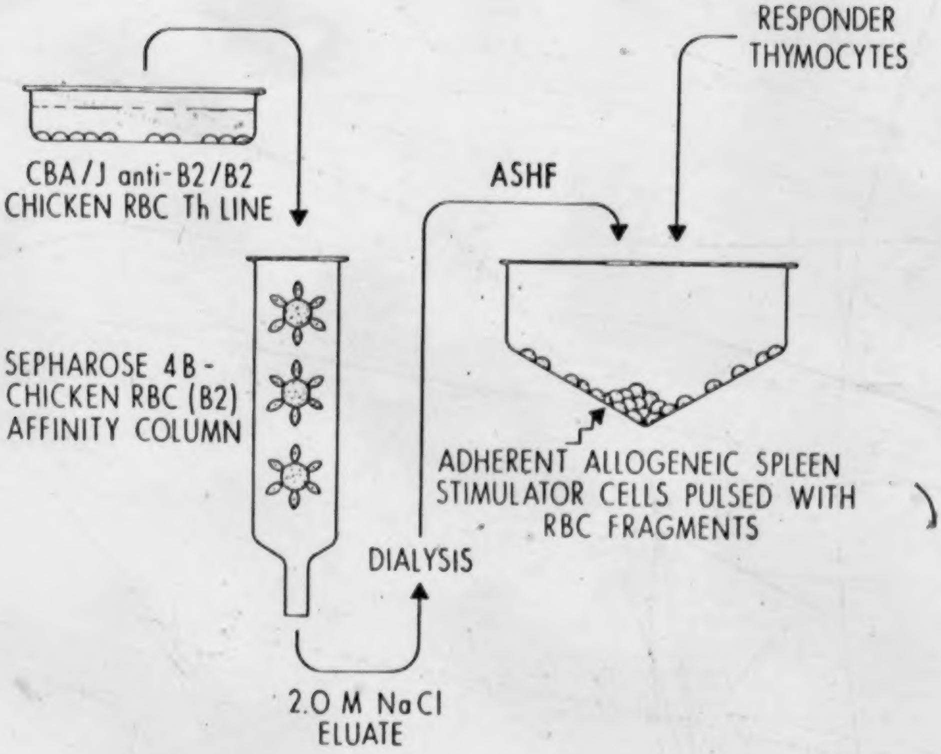

RESPONDER
THYMOCYTES
CBA/J anti-B2/B2
CHICKEN RBC Th LINE
ASHF
SEPHAROSE 4B-
CHICKEN RBC (B2)
AFFINITY COLUMN
ADHERENT ALLOGENEIC SPLEEN
STIMULATOR CELLS PULSED WITH
RBC FRAGMENTS
DIALYSIS
2.0 M NaCl
ELUATE

Figure 2: The basic experimental design for the purification and assay of ASHF is illustrated in Figure 2. Antigen-affinity purified ASHF is added to a helper-dependent assay system consisting of antigen-pulsed irradiated adherent stimulator cells and a low number of responder thymocytes. The addition of ASHF in the presence of the appropriate "carrier" antigens triggers CTLp in the responder thymocyte population to become effector CTL capable of lysing specifically target cells bearing allogeneic MHC antigens constitutively expressed on the stimulator cells.

attempted to determine if recognition of carrier determinants by the T*h* cell lines is required for the generation of cytotoxicity against the murine alloantigens on the stimulator cells. Target cells in the cytotoxicity assay do not bear the carrier antigens.

The T*h* line L61 was initially developed to provide antigen-specific help for antibody responses to CRBC of the *B*19 haplotype. Table 1 demonstrates that as few as 100 irradiated T*h* cells from line L61 can induce CTL responses from CBA thymocyte precursors in response to the BALB/c alloantigens on the (BALBc x CBA/J)F1,*B*19-pulsed adherent stimulator cells.

Cytotoxic responses were not induced in the absence of help (T*h* or IL2), or in the absence of irradiated stimulator cells. Carrier-specificity is demonstrated by the failure to generate cytotoxicity against BALB/c alloantigens in the presence of L61 cells and BRBC-pulsed stimulator cells or stimulator cells that had not been exposed to carrier erythrocyte antigens (unpulsed stimulator cells). No CTL activity was generated in the absence of thymocytes.

Table 2 demonstrates that as few as 30 CHI T*h* cells can trigger CTL responses in a carrier specific manner. A requirement for *B*2-CRBC antigens is demonstrated by the ability of CHI to generate CTL responses only in the presence of *B*2-CRBC antigens on stimulator cells, but not in the presence of unpulsed stimulators. No CTL responses were observed in the absence of responder thymocytes. Similar *B*2-CRBC-specific induction of CTL responses has been observed with clone 4C6 (unpublished observations), derived from the parent line CHI by limiting dilution.

Table 3 describes the phenotypes of the ASHF-producing T*h* line CHI and clone 4C6. Antiserum treatment and immunofluorescent analysis (not shown) demonstrated that CHI is clonally heterogeneous. This cell line includes about 40% IA$\beta$(Ia.17)$^+$ cells. Immunofluorescent analysis confirmed that 4C6 does not express detectable levels of either Lyt 1 or Lyt 2 although 4C6 cells expressed Ly7, which is selectively expressed on T*h* effector cells (123). Analysis of ASHF produced by Clone 4C6 is described in Chapter IV.

## TABLE 1

T*h* line L61 induces CTL respones in a carrier-specific manner

| Number of CBA Responder Thymocytes | Number of Stimulator Cells and Type of Carrier Antigen | Number of Irradiated L61 Cells | IL2 | % Specific Lysis |
|---|---|---|---|---|
| $10^5$ | - | - | - | <1 |
| " | - | - | + | <1 |
| " | $3x10^5$ + *B*19 FTA | - | - | <1 |
| " | " | - | + | 32.2±2.1 |
| none | " | $3x10^3$ | - | <1 |
| none | " | $3x10^2$ | - | <1 |
| none | " | $3x10^1$ | - | <1 |
| $10^5$ | " | $10^4$ | - | 28.9±1.9 |
| " | " | $3x10^3$ | - | 45.4±4.9 |
| " | " | $10^3$ | - | 14.9±5.2 |
| " | " | $3x10^2$ | - | 21.2±1.7 |
| " | " | $10^2$ | - | 8.2±2.0 |
| " | " | 30 | - | <1 |
| " | " | 10 | - | <1 |
| " | " | 3 | - | 2.1±1.3 |
| " | $3x10^5$ Unpulsed | $3x10^3$ | - | 4.4±1.1 |
| " | " | $3x10^2$ | - | <1 |
| " | " | $3x10^1$ | - | <1 |
| " | $3x10^5$+BRBC FTA | $3x10^3$ | - | <1 |
| " | " | $3x10^2$ | - | <1 |
| " | " | $3x10^1$ | - | <1 |

γ-irradiated (1500 rad) L61 cells were added to cultures containing the irradiated (1500 rad) plastic-adherent fraction from 3 x $10^5$ (BALB x CBA)F1 spleen cells that were pulsed with membrane fragments (FTA) from *B*19-CRBC, BRBC, or F1 adherent cells which had not been fed "carrier" antigens. Unpulsed stimulator cells were also cultured with an optimal concentration of IL2 as a positive control. CBA thymocyte responders ($10^5$) were added to wells as indicated. Cytotoxicity was assayed on day 5 against BALB/c Con A blast targets.

TABLE 2

*Th* line CHI induces CTL responses in a carrier-dependent manner

| Number of CBA Responder Thymocytes | Number of Stimulator Cells and Type of Carrier Antigen | Number of Irradiated L61 Cells | IL2 | % Specific Lysis |
|---|---|---|---|---|
| None | 3x10$^5$ + *B*2 FTA | 3x10$^3$ | — | <1 |
| None | " | 3x10$^2$ | — | <1 |
| None | " | 3x10$^1$ | — | <1 |
| 10$^5$ | " | — | — | 1.5±1.0 |
| " | " | — | + | 22.3±3.9 |
| " | " | 10$^4$ | — | 12.7±5.4 |
| " | " | 3x10$^3$ | — | 37.1±5.4 |
| " | " | 10$^3$ | — | 46.3±6.6 |
| " | " | 3x10$^2$ | — | 26.2±3.8 |
| " | " | 10$^2$ | — | 28.1±4.3 |
| " | " | 3x10$^1$ | — | 15.1±1.5 |
| " | " | 10$^1$ | — | <1 |
| " | 3x10$^5$ Unpulsed | 3x10$^3$ | | 1.4±2.8 |
| " | " | 3x10$^2$ | | 6.6±2.4 |
| " | " | 3x10$^1$ | | 3.7±1.8 |

Stimulator cells were pulsed with *B*2-FTA or were not exposed to "carrier" antigens (unpulsed). Culture and assay conditions were otherwise the same as described in the legend to Table I and in Chapter II.

TABLE 3

Phenotypes of ASHF-producing parental line CHI and clone 4C6

| Treatment | % Killed by Antiserum + C' | |
|---|---|---|
| | T*h* Line CHI | Clone 4C6 |
| Anti-Thy 1.2 + C' | 93.8 | 91.0 |
| Anti-Lyt 1.1 + C' | 47.0 | 2.8 |
| Anti-Lyt 2.1 + C' | 50.0 | 3.6 |
| Anti-Ia$^{k}$ + C' | 38.5 | 14.6 |

The cells used in these studies were grown in the absence of antigen or feeder cells. Procedures for antiserum treatment are described in Chapter II. Immunofluorescence assays demonstrate 4C6 to include only 3-6% Ia$^{+}$ cells.

Table 4 demonstrates that the assay system developed for detection of CRBC-specific T*h* is also able to detect T*h* that recognize soluble, non-MHC antigens such as PPD. The optimal concentration of PPD in these experiments was 50 μg/ml, although strong CTL responses were induced by $10^3$ CPPD cells cultured with 5 μg/ml PPD. The former dose of PPD is optimal for proliferation of this cell line (C. Guidos, personal communication). In this experiment, 50 μg/ml of PPD left in culture gave nearly identical results to those obtained using 50 μg/ml of pulsed antigen (not shown). Experiments are in progress to define the mechanism(s) of action by which CPPD is able to trigger thymocyte CTLp.

Tables 5 and 6 demonstrate that T*h* recognizing KLH or *Corynebacterium parvum (CP)* can trigger thymocyte CTLp in an antigen-specific manner. Table 6 demonstrates that the DBA/2J (H-$2^d$) T*h* line D*CP* can effectively induce allogeneic CBA/J (H-$2^k$) CTLp in an antigen-specific manner. Other experiments have demonstrated that D*CP* is equally capable of triggering H-2-identical BALB/c thymocyte CTLp in the presence of the *CP* and allogeneic (BALB/c x CBA/J)F1 stimulator cells (unpublished observations).

The previous tables demonstrate the adaptability of the system developed in these studies to detect antigen-specific T*h* that are able to trigger CTLp. It has been be used to detect T*h* that recognize either membrane-bound murine MHC antigens (123), soluble CRBC membrane fragments (Tables 1 and 2), or soluble nominal antigens such as KLH, *CP*, or PPD (Tables 4-6 and unpublished observations). This assay detects T*h* participating in CTLp induction that may be different from T*h* subsets that secrete IL2 and/or proliferate in response to antigen.

The carrier-specific induction of CTL by a variety of T*h* (Tables 1,2,4-6) is clearly different from the antigen non-specific IL2-dependent induction of CTLp. However, it was necessary to determine if these CTL responses resulted from the release of antigen-specific factors by the T*h* or from an antigen-specific induction of IL2 production by the T*h*. This was most easily achieved by testing the helper activity of affinity-purified, IL2-free material secreted by T*h* cell lines.

## TABLE 4

*Th* line CPPD induces CTL responses in a carrier-dependent manner

| Concentration of PPD Used for Pulsing Stimulators | Number of Irradiated CPPD Cells | IL2 | % Lysis of BALB/c Targets |
|---|---|---|---|
| — | — | — | <1 |
| — | — | + | 25.5±2.2 |
| — | $10^3$ | — | <1 |
| — | $10^2$ | — | <1 |
| — | $10^1$ | — | <1 |
| 5 μg/ml | — | — | <1 |
| " | — | + | 33.0±7.6 |
| " | $10^3$ | — | 27.3±6.1 |
| " | $10^2$ | — | <1 |
| " | $10^1$ | — | <1 |
| 50 μg/ml | — | — | <1 |
| " | — | + | 37.7±2.9 |
| " | $10^3$ | — | 21.2±4.0 |
| " | $10^2$ | — | 17.5±7.4 |
| " | $10^1$ | — | <1 |

Three x $10^5$ (BALB/c x CBA/J)F1 spleen cells were incubated with 0.1 ml MEM or with MEM containing the indicated concentrations of PPD. After 2 hours, non-adherent cells and unbound antigen were removed. Stimulators were γ-irradiated (1500 rad) prior to the addition of medium, IL2, or irradiated *Th* (1500 rad) and 1 x $10^5$ CBA/J thymocytes. Total culture volume was 0.2 ml. Cytotoxicity was assayed after 5 days against BALB/c targets as described in Chapter II.

## TABLE 5

T*h* line CKA induces CTL responses in a carrier-specific manner

| Type and Concentration of "Carrier" Used for Pulsing Stimulators | Number of Irradiated CKA Cells | IL2 | % Lysis of BALB/c Targets |
|---|---|---|---|
| PPD(50 μg/ml) | — | — | $1.0\pm1.5$ |
| " | — | + | $55.1\pm3.0$ |
| " | $3x10^4$ | — | $1.0\pm1.0$ |
| " | $3x10^3$ | — | $2.7\pm1.8$ |
| " | $3x10^2$ | — | <1 |
| " | 30 | — | <1 |
| KLH(2.5 μg/ml) | — | — | <1 |
| " | — | + | $31.0\pm3.5$ |
| " | $3x10^4$ | — | $21.0\pm2.8$ |
| " | $3x10^3$ | — | $17.7\pm3.9$ |
| " | $3x10^2$ | — | $6.2\pm4.1$ |
| " | 30 | — | <1 |
| KLH(25 μg/ml) | — | — | $2.1\pm0.5$ |
| " | — | + | $60.3\pm2.8$ |
| " | $3x10^4$ | — | $30.0\pm1.4$ |
| " | $3x10^3$ | — | $25.4\pm3.3$ |
| " | $3x10^2$ | — | $13.8\pm2.5$ |
|  | 30 |  | <1 |

Three x $10^5$ (BALB/c x CBA/J)F1 spleen cells were cultured with the indicated concentrations of PPD or KLH, and KLH-reactive CKA T*h*, IL2 or medium. One x $10^5$ CBA/J thymocytes were added to each culture well. Culture and assay conditions for the detection of cytotoxicity against $^{51}$Cr-labelled BALB/c target cells are described in Chapter II and in the legend to Table 4.

TABLE 6

T*h* line D*CP* triggers CTL responses in a carrier-dependent manner

| Protein Concentration of *CP* Used for Pulsing Stimulators | Number of Irradiated D*CP* Cells | IL2 | % Lysis of BALB/c Targets |
|---|---|---|---|
| — | — | — | <1 |
| — | — | + | 35.8±5.0 |
| — | $10^3$ | — | <1 |
| — | $10^2$ | — | <1 |
| — | 10 | — | <1 |
| *CP*(7 μg/ml) | — | — | <1 |
| " | — | + | 48.4±8.6 |
| " | $10^3$ | — | 21.5±3.6 |
| " | $10^2$ | — | 10.9±1.9 |
| " | 10 | — | <1 |
| *CP*(70 μg/ml) | — | — | <1 |
| " | — | + | 44.7±4.6 |
| " | $10^3$ | — | 31.7±6.7 |
| " | $10^2$ | — | 6.0±4.7 |
| " | 10 | — | 5.2±2.9 |

Three x $10^5$ (BALB/c x CBA/J)F1 stimulator cells were pulsed with the indicated concentrations of *Corynebacterium parvum (CP)* or were not exposed to "carrier" antigens. Culture and assay conditions were the same as described in the legend to Figure 4 and in Chapter II.

### Antigen-Affinity Purified Helper Factors Induce CTL in an Antigen-Specific Manner

In order to detect ASHF activity in culture supernatants that were known to contain IL2, it was essential to separate non-specific lymphokines from ASHF by affinity-chromatography as described in Chapter II and confirmed later in this section. ASHF was purified from the T*h* line CHI that recognizes determinants on CRBC of the *B*2 haplotype (Table 2).

Table 7 demonstrates that ASHF from the T*h* line CHI is able to induce CTL responses in a carrier-specific manner. Cytotoxic responses were not induced in the absence of help. As little as 50 nanograms of ASHF-containing protein induces significant CTL responses against BALB/c antigens (hapten determinants) if the *B*2-CRBC FTA (carrier determinants) are presented on the stimulator cells during culture. Negligible responses were generated when the stimulator cells did not present the *B*2-CRBC carrier determinants recognized by the ASHF.

Cytotoxic responses were also induced in the presence of IL2 although, as expected, carrier-specificity was not demonstrated in IL2-mediated induction of CTLp. In this experiment and many, but not all others, maximal induction by ASHF greatly exceeded that generated by optimal concentrations of IL2. All test groups in this experiment were also assayed for their ability to lyse B10 targets. Lysis of B10 targets was less than 1% in all groups. This demonstrates that ASHF is not a polyclonal activator and will trigger only CTLp that are also recognizing antigen. Further characterization of the specific recognition of target cells by ASHF-induced CTL is presented in Chapter V.

Antigen-affinity chromatography is routinely employed for the purification of ASHF (239,290,294). In order to further verify the antigen-specificity of ASHF we attempted to determine if ASHF from the T*h* line CHI, that recognizes *B*2-CRBC antigens (Table 2, and Ref. 239,294) would show preferential binding to a *B*2-CRBC affinity column. CHI supernatants were first applied to a *B*13-CRBC-Sepharose column. The effluent from this *B*13-CRBC column was next run through a *B*2-CRBC-Sepharose column. Both columns were eluted with 2M NaCl. The eluates were dialyzed and tested for ASHF and IL2 activities.

## TABLE 7

Affinity purified helper factors induce CTL responses in a carrier-specific manner

| Final Dilution (ng protein) of CHI-ASHF | IL2 | % Specific Lysis of BALB/c Targets. | | |
|---|---|---|---|---|
| | | Stimulator Cells Pulsed with: | | |
| | | *B*2-FTA | *B*13-FTA | No Carrier |
| 1/10 ($5x10^4$) | — | 27.6±7.2 | <1 | <1 |
| 1/100 ($5x10^3$) | — | 83.8±5.4 | 9.2±4.4 | <1 |
| $1/10^3$ (500) | — | 47.3±8.2 | <1 | <1 |
| $1/10^4$ (50) | — | 18.3±8.2 | <1 | <1 |
| $1/10^5$ (5) | — | 11.1±7.9 | <1 | <1 |
| $1/10^5$ (0.5) | — | <1 | <1 | <1 |
| — | — | <1 | <1 | <1 |
| — | + | 18.3±7.7 | 43.2±8.5 | 39.6±9.1 |

The plastic-adherent fraction from 3 x $10^5$ (BALB X CBA)F1 spleen cells was pulsed with *B*2-CRBC FTA,or *B*13-CRBC FTA prior to removal of excess antigen by washing. Subsequently the stimulator cells were irradiated (1500 rad). Dilutions of ASHF, IL2, or medium and $10^5$ CBA responder thymocytes were added to each well. Percent specific lysis of BALB/c or B10 targets was assayed. Lysis of B10 targets was less than 1% (not shown).

Figure 3 demonstrates that only the molecules in the CHI supernatant that ran through the *B*13 column and subsequently bound to the *B*2-CRBC affinity column were able to induce CTL responses over a wide dilution range.

Only background CTL responses were induced in the absence of *B*2-bound ASHF or in the presence of the *B*13-bound material. The results of these experiments demonstrate that antigen-affinity purified ASHF from the T*h* line CHI is antigen-specific both in its ability to bind nominal antigen and in its requirement for nominal antigen in the induction of CTLp. The induction of CTL responses from BALB/c thymocytes in this experiment also demonstrates that *B*2-bound CHI-ASHF is able to trigger CTLp in an H-2 unrestricted manner. This point will be discussed in greater detail.

## ASHF Does Not Contain Any IL2 Activity

Table 7 and Figure 3 demonstrate that ASHF is clearly different from IL2 by virtue of its carrier specificity. However, we also wished to determine if ASHF had any IL2 activity, as defined by the ability to support the survival of an IL2-dependent CTL line. Figure 4 demonstrates that the *B*2-bound ASHF preparation that triggered the induction of CTLp over a wide dilution range (Figure 3), contained no IL2 activity.

Indicator cells incubated with medium which did not contain any IL2 exhibited the same low levels of metabolic activity as did cells which were cultured with any dilution of *B*2-bound or *B*13-bound ASHF. In contrast, our partially-purified IL2 preparation stimulated proliferation of the indicator cells over a wide dilution range of IL2. Similar experiments with other preparations of ASHF demonstrated negligible levels of IL2 activity, as determined by $^{125}$IUdR or $^{3}$H-thymidine incorporation (Ref. 294 and unpublished observations).

## $Ca^{++}$-Sufficient ASHF is Not H-2 Restricted in the Recognition of Antigen-Pulsed Stimulator Cells

Figure 3

ASHF bind specifically to nominal antigen

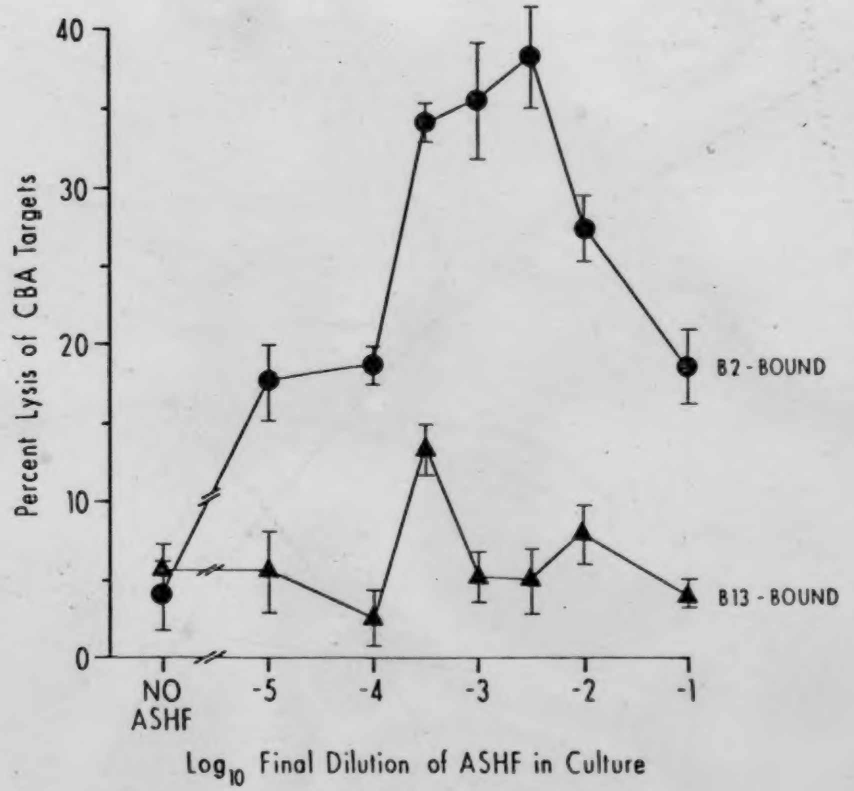


Figure 3: Supernatant from the T*h* line CHI was first passed through an antigen-affinity column consisting of *B*13-CRBC-Sepharose 4B, prepared as described (290). The unbound effluent was next run through an antigen-affinity column consisting of *B*2-CRBC-Sepharose-4B. The 2M NaCl eluate from each column was dialyzed against PBS and concentrated. The protein concentrations of the undiluted *B*2-bound and *B*13-bound ASHF fractions were 1.80 and 1.95 mgs/ml, respectively. The *B*2-bound(●) and *B*13-bound (▲) fractions were assayed for their ability to induce CTL responses in cultures containing $10^5$ BALB/c responder thymocytes and 3 x $10^5$ CBA/J stimulator cells that were pulsed with *B*2 CRBC FTA. Cytotoxicity was assayed against CBA/J targets.

Figure 4

ASHF does not contain detectable IL2 activity

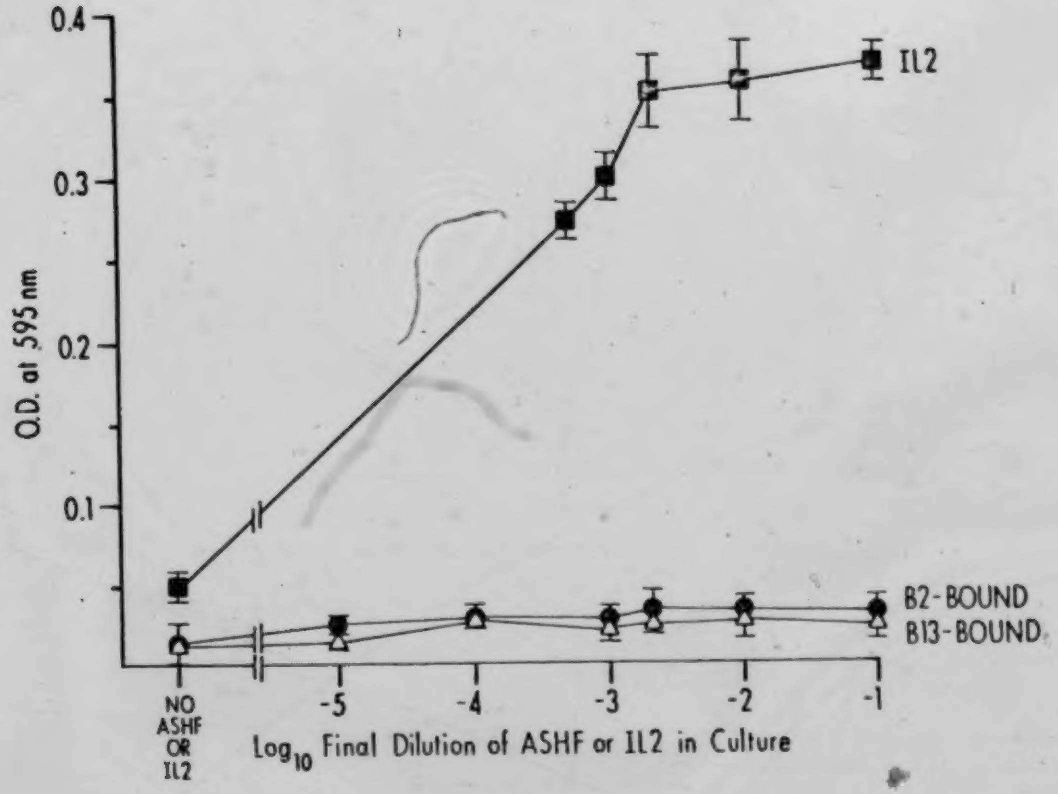


Figure 4: B2-bound and *B*13-bound ASHF preparations (see Fig. 3 andlegend) were titrated for IL2 activity. Log$_{10}$ dilutions of *B*2-bound ASHF (•), *B*13-bound ASHF (▵), or partially-purified IL2 (■) were assayed for their ability to support the survival of an IL2-dependent murine CTL line (324) using the MTT assay system (325 and Chapter II). Each point represents the mean of six individually assayed cultures ± one standard deviation.

Our initial experiments were performed with ASHF, purified under $Ca^{++}$-sufficient conditions, in order to insure that the functional association of ASHF subunits (294) was maintained in the ASHF preparations tested for helper activity. Because of the uncertainties regarding H-2 restricted collaborative interactions leading to the induction of CTLp (29), we wished to determine if CTL induction by ASHF is restricted at the level of the adherent cells. The lack of H-2 restriction between $Ca^{++}$-sufficient ASHF (as defined by the phenotype of the ASHF-producing cells) and adherent stimulator cells is demonstrated in Table 8.

$Ca^{++}$-sufficient ASHF produced by the CBA ($H\text{-}2^{k}$) cell line CHI can trigger CBA CTLp in response to *B2*-pulsed BALB/c adherent stimulator cells (line 3) or *B2*-pulsed B10 adherent stimulator cells (line 15). Carrier-specificity of ASHF is again demonstrated in this experiment by the failure of the *B2* CRBC-specific ASHF to trigger CTLp in response to *B*13-pulsed adherent stimulator cells (lines 6,12, and 18). In contrast to the carrier-specificity of ASHF, IL2 induced similar levels of CTL activity from thymocytes in response to *B2*-pulsed or *B*13-pulsed stimulator cells (lines 2 & 5, 8 & 11, 14 & 17). It is possible that carrier antigen (CRBC-FTA) released from allogeneic (non-CBA) adherent stimulator cells could have been re-presented by CBA adherent cells derived from the responder thymocyte population, thus making it appear that $Ca^{++}$-sufficient ASHF is not H-2 restricted at the level of the adherent cell (lines 3 and 15). This possibility is excluded by the ability of this ASHF preparation to induce CTL responses in a carrier specific-manner when both the responder thymocytes (BALB/c) and adherent stimulator cells (B10) were allogeneic to the ASHF (lines 7-12).

## $Ca^{++}$-Sufficient ASHF Is Not H-2 Restricted in Its Ability to Interact with CTLp

Figure 3 and Table 8 demonstrate that H-2 restriction at the level of cell interactions, which has been reported to occur betweeen $Ca^{++}$-sufficient ASHF and the precursors of antibody-secreting B lymphocytes (288,290), does not occur in the collaboration between ASHF and CTLp.

## TABLE 8

Ca$^{++}$ - sufficient CHI-ASHF is not H-2 restricted

| | 3x10$^5$ Responder Thymocytes | CRBC-FTA Pulsed Stimulators | CHI-ASHF | IL2 | % Specific Lysis |
|---|---|---|---|---|---|
| 1 | CBA | BALB-*B*2 | — | — | <1 |
| 2 | " | " | — | + | 37.2±4.1 |
| 3 | " | " | + | — | 27.3±4.5 |
| 4 | " | BALB-*B*13 | — | — | <1 |
| 5 | " | " | — | + | 34.8±5.7 |
| 6 | " | " | + | — | <1 |
| 7 | BALB | B10-*B*2 | — | — | <1 |
| 8 | " | " | — | + | 57.4±3.8 |
| 9 | " | " | + | — | 19.5±4.8 |
| 10 | " | B10-*B*13 | — | — | <1 |
| 11 | " | " | — | + | 54.3±3.3 |
| 12 | " | " | + | — | <1 |
| 13 | CBA | B10-*B*2 | — | — | <1 |
| 14 | " | " | — | + | 44.4±7.4 |
| 15 | " | " | + | — | 43.7±4.9 |
| 16 | " | B10-*B*13 | — | — | <1 |
| 17 | " | " | — | + | 39.1±4.2 |
| 18 | " | " | + | — | <1 |

Three x 10$^5$ BALB/c or CBA/J thymocytes were cultured with either *B*2- or *B*13-FTA-pulsed adherent stimulator cells from the mouse strains indicated and CHI-ASHF(4 $\mu$g ASHF protein/culture) or an optimal concentration of IL2. Cytotoxicity was assayed against Con A-induced blasts syngeneic to the stimulator cells. A titration of ASHF, included in this experiment, demonstrated that as little as 0.4 $\mu$g of Ca$^{++}$-sufficient ASHF was still effective in triggering CTLp in an antigen-specific manner, but was not H-2 restricted in its interaction with either CTLp or adherent stimulator cells (APC) (not shown).

Table 8 demonstrates that ASHF purified in the presence of $Ca^{++}$ is able to induce CTL responses from syngeneic (CBA) or allogeneic (BALB/c) thymocytes in an antigen-specific manner. CTL responses were not induced from either BALB/c or CBA thymocytes cultured with *B*13-FTA-pulsed B10 or *B*13-FTA-pulsed BALB adherent stimulator cells in the presence of *B*2-CRBC-specific CHI-ASHF. Positive CTL responses generated against *B*13-pulsed stimulator cells in the presence of IL2 demonstrated that the *B*13 carrier antigens did not inhibit the generation of CTL responses against murine MHC alloantigens (Table 8 - lines 5,11, and 17). $Ca^{++}$-sufficient CHI-ASHF can induce CTL from BALB/c thymocytes in response to FTA-pulsed CBA stimulators in a carrier-specific manner (Figure 3 and Table 9). $Ca^{++}$-sufficient ASHF is also able to induce CTL responses in a carrier specific manner when both the thymocyte CTLp and adherent cells are allogeneic to the ASHF-producing cells (Table 8 - lines 9 and 12). The results of these experiments demonstrate that $Ca^{++}$-sufficient ASHF is antigen-specific but does not exhibit any requirement for identity with either CTLp or adherent cells in order to trigger CTLp.

### $Ca^{++}$-Deficient ASHF Requires Syngeneic Adherent Cells for the Induction of CTLp

Previous studies on the requirement for $Ca^{++}$ in the functional association of ASHF subunits (292) suggested that ASHF eluted from antigen-affinity columns after treatment with EGTA, which chelates $Ca^{++}$, may exhibit altered inductive activity or H-2 restriction properties in the induction of CTLp. BALB/c mice were used as a source of responder thymocytes to eliminate the possibility that adherent cells derived from a thymocyte population expressing $H\text{-}2^k$ -encoded products might obscure a requirement for syngeneic stimulator cells. Table 9 demonstrates that CHI-ASHF, purified in the presence or absence of $Ca^{++}$, are able to induce similar levels of CTL activity from BALB/c thymocytes responding to CRBC-pulsed CBA adherent stimulator cells.

Both $Ca^{++}$-sufficient and $Ca^{++}$-deficient CHI-ASHF are able to trigger allogeneic (BALB/c) CTLp in the presence of adherent cells bearing the carrier determinants recognized

TABLE 9

H-2 restriction properties of CHI-ASHF purified in the presence or absence of calcium

| Strain Combination | ASHF Dilution | IL2 | % Specific Lysis | | | |
|---|---|---|---|---|---|---|
| | | | Ca$^{++}$- sufficient ASHF | | Ca$^{++}$-deficient ASHF | |
| | | | Stimulators pulsed with: | | | |
| | | | *B*2-FTA | *B*13-FTA | *B*2-FTA | *B*13-FTA |
| BALB anti-CBA | — | — | 1.3±1.8 | <1 | <1 | <1 |
| | — | + | 14.5±2.6 | 25.6±3.4 | 14.4±3.7 | 17.2±1.9 |
| | 1/25 | — | 26.8±1.8 | 4.3±2.0 | 20.6±1.7 | 4.6±2.9 |
| | 1/50 | — | 28.3±3.1 | <1 | 20.0±2.4 | 4.6±1.8 |
| | 1/100 | — | 25.9±3.8 | 4.9±3.7 | 21.0±3.1 | <1 |
| | 1/200 | — | 23.7±2.4 | 3.7±1.3 | 21.8±2.5 | <1 |
| | 1/800 | — | 17.5±3.0 | <1 | 17.4±2.8 | <1 |
| BALB anti-B10 | — | — | <1 | <1 | <1 | NT |
| | — | + | 25.7±1.9 | 23.7±4. | 18.6±3.1 | NT |
| | 1/25 | — | 23.3±1.8 | <1 | 5.4±3.7 | NT |
| | 1/50 | — | 24.0±2.4 | <1 | <1 | NT |
| | 1/100 | — | 28.0±2.5 | <1 | <1 | NT |
| | 1/200 | — | 20.1±1.9 | <1 | <1 | NT |
| | 1/800 | — | <1 | <1 | <1 | NT |

Half of a Ca$^{++}$-sufficient CHI-ASHF was rechromatographed to produce Ca$^{++}$-deficient ASHF as described in Chapter II. Dilutions of ASHF preparations were added to cultures containing $10^5$ BALB/c thymocytes and 3 x $10^5$ CBA/J or B10 stimulator cells that had been pulsed with *B*2-FTA or *B*13-FTA. A 1/800 dilution corresponds to 5.2 and 3.1 $\mu$g of Ca$^{++}$-sufficient and Ca$^{++}$-deficient ASHF-containing protein, respectively. Cytotoxicity was assayed against unpulsed target cells syngeneic to the stimulator cells. NT = not tested.

by the ASHF. As in previous experiments, this triggering is carrier-specific. Both $Ca^{++}$-sufficient and $Ca^{++}$-deficient ASHF preparations only induced CTL responses in the presence of *B*2-pulsed but not *B*13-pulsed adherent CBA stimulator cells (Tables 8 & 10).

When the activity of ASHF purified in the presence or absence of $Ca^{++}$ are compared in cultures where both the adherent stimulator cells and the responder thymocytes are allogeneic to the ASHF-producing cells (BALB anti-B10), only the $Ca^{++}$-sufficient ASHF preparation is able to induce CTL responses. In this completely allogeneic combination, the induction of CTLp by $Ca^{++}$-sufficient ASHF preparations requires only the carrier determinants (*B*2-CRBC) recognized by the ASHF in addition to the "hapten" determinants (murine MHC antigens) recognized by the CTLp. In contrast, the $Ca^{++}$-deficient ASHF requires syngeneic adherent-cell products in addition to the hapten and carrier determinants recognized by the CTLp and ASHF, respectively, in order to effectively trigger CTLp.

### H-2-Encoded Gene Products Are Required to Restore the Ability of $Ca^{++}$-Deficient ASHF to induce CTLp

In order to define the genetic regions which encode the adherent cell products required for the induction of CTLp by $Ca^{++}$-deficient ASHF, congenic mice were used as the source of adherent stimulator cells. Table 10 demonstrates that CTL responses were induced from BALB/c thymocytes cultured with $Ca^{++}$-deficient ASHF (of CBA origin) and B2-pulsed stimulator cells from CBA or B10.BR mice, but not B10 or B10.A(5R) mice. Carrier-specificity is demonstrated by the failure to induce CTL responses to *B*13-pulsed stimulator cells by ASHF in all strain combinations tested. The ability of CBA or B10.BR but not B10 stimulator cells to restore the CTL-inductive activity of $Ca^{++}$-deficient ASHF indicates that H-2-encoded products of adherent cells are required in the induction of CTLp by ASHF. The inability of B10.A(5R) adherent stimulator cells to restore the activity in this experiment suggests that products of the I-J and/or I-E subregions are not sufficient in this capacity.

TABLE 10

H-2 encoded gene products are required to restore the ability of $Ca^{++}$ deficient ASHF to induce CTL

| Responder Thymocytes | FTA-Pulsed Adherent Stimulators | ASHF Dilution | %Specific Lysis Stimulators pulsed with: *B*2-FTA | *B*13-FTA |
|---|---|---|---|---|
| BALB/c | CBA | $10^{-1}$ | 19.0±3.9 | 2.0±2.0 |
| " | " | $10^{-2}$ | 12.2±5.4 | <1 |
| " | " | $10^{-3}$ | 2.1±2.0 | <1 |
| " | " | — | <1 | <1 |
| " | B10 | $10^{-1}$ | 5.0±2.4 | <1 |
| " | " | $10^{-2}$ | <1 | <1 |
| " | " | $10^{-3}$ | <1 | <1 |
| " | " | — | 3.5±1.0 | <1 |
| " | B10.BR | $10^{-1}$ | 22.0±2.0 | <1 |
| " | " | $10^{-2}$ | 8.3±5.0 | <1 |
| " | " | $10^{-3}$ | <1 | <1 |
| " | " | — | <1 | <1 |
| " | B10.A(5R) | $10^{-1}$ | <1 | <1 |
| " | " | $10^{-2}$ | <1 | <1 |
| " | " | $10^{-3}$ | <1 | <1 |
| " | " | — | <1 | <1 |

Dilutions of $Ca^{++}$-deficient ASHF from the cell line CHI, medium, or an optimal concentration of IL2 were added to cultures containing 3 x $10^5$ BALB/c thymocytes and the irradiated plastic-adherent fraction from 3 x $10^5$ spleen cells of the strains of mice indicated. These stimulator cells were pulsed with either FTA from *B*2 CRBC (*B*2-FTA) or *B*13 CRBC (*B*13-FTA). Cytotoxicity was assayed on day 5 against unpulsed targets syngeneic to the stimulator strain. Significant cytotoxicity was generated in all responder and FTA-pulsed stimulator cell combinations cultured in the presence of IL2 (data not presented).

H-2-encoded products derived from adherent cells appear to be required as "cofactors" necessary for the induction of CTLp by $Ca^{++}$-deficient ASHF. However, these "cofactors" are not essential for the binding of $Ca^{++}$-sufficient or $Ca^{++}$-deficient ASHF to nominal antigen (CRBC) on antigen-affinity columns.

## D. Discussion

In the experiments presented in this chapter, soluble factors are able to replace intact T*h* in an antigen-specific manner. Antigen-specific recognition of carrier determinants by ASHF is reflected in the ability of ASHF secreted by a *B*2-specific T*h* line to bind to *B*2-CRBC but not B13-CRBC on antigen-affinity columns (Fig. 3). The requirement for recognition of carrier antigen in the induction of CTLp is demonstrated by the ability of ASHF preparations to induce CTL responses to adherent stimulator cells pulsed with *B*2-CRBC antigens but not to unpulsed stimulator cells or stimulator cells which were pulsed with *B*13-CRBC antigen (Tables 7-10).

The requirement for recognition of murine MHC antigens by CTLp during ASHF-mediated induction demonstrates that ASHF is not a polyclonal activator of CTLp. Binding of antigen to CTR may induce the expression of ASHF receptors or otherwise induce changes that facilitate the inductive effects of ASHF. The mechanism by which ASHF induces CTLp in our assay system is unclear. Antigen-specific helper factors may be acting directly on CTLp or may act indirectly via the induction of antigen-specific T*h* precursors which then interact with CTLp. Further experiments are necessary to define fully the mechanism of CTLp induction by ASHF.

The use of antigen affinity-purified ASHF preparations that are free of IL2 activity (Fig. 4, Ref. 294, and unpublished observations) facilitates the analysis of H-2 restricted events leading to CTL induction. In *in vitro* systems, lymphokine preparations containing IL2 are likely to bypass or obscure requirements for H-2 restricted collaborative interactions, as discussed previously (Ref. 29 and Chapter I). H-2 restriction in the induction of CTLp by

ASHF could operate at two possible levels: *a)* at the level of antigen recognition whereby nominal antigen can only be recognized by T*h*-derived ASHF when it is presented in the context of MHC-encoded molecules or *b)* at the level of cell surface interaction molecules such that either identical complementarity (A/A) or non-identical complementarity (A/B) is required between the collaborating cell types. One might expect that either or both of these types of mechanisms may be operating in the induction of CTLp (29).

Binding of ASHF to antigen-affinity columns demonstrates that presentation of CRBC antigens in the context of MHC-encoded molecules is apparently not essential for the binding of ASHF to nominal antigen. Many other laboratories have also reported the binding of ASHF to nominal antigens that appear not to be presented in the context of murine MHC molecules (286-294). Independent of whether or not ASHF recognition of antigen is H-2 restricted, our results imply that H-2 encoded molecules from adherent cells are required at other steps of CTLp induction by ASHF that do not involve recognition of nominal antigen.

Tables 9 and 10 provide functional evidence that $Ca^{++}$-deficient ASHF preparations require H-2-encoded products from adherent cells that are syngeneic to the ASHF-producing cells in order to trigger CTL responses. $Ca^{++}$-deficient ASHF appears to be functionally "incomplete". In contrast, prior to EGTA treatment, the $Ca^{++}$-sufficient ASHF is H-2 unrestricted. These results strongly suggest that H-2-encoded molecules present on "complete" ($Ca^{++}$-sufficient) ASHF are either removed or functionally inactivated by EGTA treatment. Binding of ASHF to nominal antigen is not affected by EGTA treatment. Experiments are in progress to define the H-2-encoded adherent cell products that are required to reconstitute the antigen-specific CTL-inductive activity of $Ca^{++}$-deficient ASHF. The previous report of Ia determinants on ASHF participating in the induction of CTL responses to tumor antigens (271) can be interpreted to suggest that Ia antigens may be the moities provided by syngeneic adherent cells that are required to reconstitute the functional activity of $Ca^{++}$-deficient (incomplete) ASHF.

The clonally heterogeneous ASHF-producing cell line CHI includes a subpopulation of cells that express Ia antigens (Table 3). It is suggested that Ia positive cells in the cell line CHI provide the H-2-encoded antigen-nonspecific subunit(s) of a "complete" ($Ca^{++}$-sufficient) ASHF that are either removed or functionally inactivated by EGTA treatment. It appears that complementarity between "incomplete" ASHF and H-2-encoded molecules is the basis for the H-2 restriction pattern we have reported.

These results with ASHF do not conflict with observations on the effects of antigen-nonspecific lymphokines in CTLp induction. It is suggested that prior to the expression of receptors for non-specific lymphokines, CTLp may require antigen-specific signals (ASHF).

In conclusion, the results of these experiments suggest an H-2 restricted molecular interaction in the induction of CTLp by ASHF. The "incomplete" ($Ca^{++}$-deficient) ASHF acquires functional capacity only when appropriate "completor" molecules are provided by syngeneic adherent cells. The T*h*-derived antigen-binding moiety and antigen-nonspecific H-2-encoded product(s) appear to be assembled extracellularly to form "complete" ASHF. This is similar to antigen-specific suppressor factors which appear to consist of multiple polypeptide subunits, produced in some cases by different cell types (316,317). It has been proposed that interaction occurs between a "constant region" of the antigen-binding "incomplete" ASHF produced by a T*h* cell and H-2-encoded products derived only from syngeneic adherent cells (29). It is suggested that ASHF plays a central role in the primary induction of both B and T lymphocyte responses. Further functional and molecular characterization of ASHF participating in the induction of CTLp is described in the following chapters.

# IV. DISTINCT ANTIGEN-SPECIFIC T CELL-DERIVED HELPER FACTORS ARE REQUIRED FOR THE INDUCTION OF CYTOTOXIC T CELL AND B CELL RESPONSES

## A. Introduction

The immune system uses a diverse arsenal of effector mechanisms to eliminate pathogenic microorganisms from the body. This includes cell-mediated effectors such as CTL, DTH T cells, and natural killer (NK) cells, as well as 5 functionally and biochemically distinct isotypes of immunoglobulins. To be efficient, the immune system must not merely provide "a response" but must appropriately regulate the class of response induced. Many uncertainties exist regarding the ability of both antigen-specific and antigen-nonspecific stimuli in the preferential induction of T or B lymphocyte responses. This chapter will focus on the role of ASHF in the selective induction of CTL or IgM responses.

Experiments described in this thesis and elsewhere demonstrate that ASHF from the T*h* line CHI trigger both CTL and primary IgM responses *in vitro* (29,239,294). To compare the *B*2-CRBC-specific induction of CTLp with the *B*2-CRBC-specific induction of IgM responses, we have used the helper-dependent CTL induction assay described in the previous chapters and elsewhere (29,239). Although CHI-derived ASHF triggers both CTL and IgM responses in an antigen-specific manner, it was not known if our ASHF preparations contained two types of class-specific ASHF or if the same ASHF were responsible for triggering both CTL and IgM responses. This type of comparative analysis has never been previously reported. By anion-exchange chromatography of CHI-derived, antigen-affinity purified supernatant, we have been able to separate the ASHF that trigger CTLp (ASHF-*CTL*) from the ASHF that trigger B cell IgM responses (ASHF-*B*). In addition, we were able to show that ASHF purified from clone 4C6 (derived from the previously described T*h* line CHI) efficiently triggers CTLp but does not specifically induce AFC responses, thus providing further evidence that ASHF-*CTL* and ASHF-*B* are distinct and separable entities. The roles of these distinct forms of ASHF in the regulation of the class of immunity induced in response to antigenic challenge will be discussed. It is suggested that antigen-nonspecific subunits of ASHF may influence its ability to preferentially induce CTL or B cell responses.

## B. Materials and Methods

*Antigens.* In addition to the antigens described in Chapter II, lipopolysaccharide (LPS) from *E. coli* serotype O55.B5 (Sigma Chemical Co., St. Louis, MO) was used.

*Ion Exchange Chromatography.* Antigen-affinity purified material was eluted from a DEAE-Sephacel column (1x9 cm - Pharmacia, Uppsala) by a linear concentration gradient of NaCl (0 to 0.5 M) in 0.05 M HEPES buffer (pH 7.5) (294). All fractions were dialysed exhaustively against PBS before being assayed for helper activity in the induction of CTL or IgM responses. The DEAE elution profile of CHI-ASHF is illustrated in Figure 5. Protein concentrations were determined by absorbancy at 280 nm using bovine serum albumin as a standard. The total amount of protein recovered in the antigen-affinity bound material was 8.6 mg. Recovery of protein from DEAE-Sephacel chromatography was 75%. All steps of anion-exchange chromatography were performed by Dr. C. Shiozawa.

*Assay for Antigen-Specific Help for B Cells.* Enrichment of CBA/J splenic B cells that recognize CRBC antigens by rosetting and culture techniques have been described previously (290). Briefly, mononuclear spleen cells were purified on an Isopaque-Ficoll (Pharmacia Fine Chemicals Inc. - Uppsala, Sweden) gradient prior to washing and incubation with B2 or B13 CRBC. After incubation, rosette-forming cells (RFC) and CRBC were separated from non-rosetted cells (NR) on an Isopaque-Ficoll gradient. After washing, 2 x $10^3$ RFC, 1 x $10^6$ irradiated (1500 rad) NR, and ASHF, LPS, or medium were cultured in Linbro microculture plates in Click's medium with 5% FCS. As described previously, NR provide antigen-nonspecific differentiation factors for RFC (290). The total volume in each microculture well was 0.2 ml. Cultures were tested after four days for the presence of antibody forming cells (AFC) by the direct hemolytic plaque assay of Cunningham and Szenberg (331) as described previously (290). All AFC assays were performed by Dr. C. Shiozawa. IgM responses are expressed as AFC ± S.E..

## C. Results

### Triggering of both CTL and IgM Responses by ASHF

Table 11 demonstrates that antigen-affinity purified supernatants from the cell line CHI are able to induce helper-dependent primary CTL responses and primary IgM responses *in vitro* in an antigen-specific manner. Table 11 demonstrates that significant CBA/J (H-$2^k$) CTL responses against BALB/c (H-$2^d$) alloantigens are induced by ASHF only when the (BALB/c x CBA)F1 stimulator cells are pulsed with *B*2-CRBC FTA, but not when stimulator cells are pulsed with *B*13-CRBC FTA. Previous reports demonstrate that antigen-affinity purified ASHF contain no detectable IL2 activity (239,294).

The same preparation of ASHF is also able to preferentially induce primary IgM responses to *B*2-CRBC (Table 11). Co-purification of antigen-nonspecific B cell-inductive lymphokines with ASHF may explain the weak triggering of AFC reactive with *B*13-CRBC. This nonspecific triggering never exceeded 33% of LPS-induced responses to *B*13-CRBC. Significant nonspecific activity was not detectable at lower concentrations that were able to induce significant *B*2-CRBC AFC responses. The ability of CHI-ASHF to induce CTL or AFC responses in an antigen-specific manner has been confirmed in many experiments as reported elsewhere (29,239,294).

### Separation of Antigen-Specific Helper Factor Activities for the Induction of CTL and IgM Responses by Ion-Exchange Chromatography

To determine if the CTL and IgM responses were induced by two distinctly different ASHF in the antigen-affinity purified supernatants from the cell line CHI, the *B*2-specific ASHF from this cell line were subjected to ion-exchange chromatography. Pooled fractions were tested for antigen-specific helper activity in the generation of CTL and AFC. The elution

## TABLE 11

Antigen-Affinity Purified ASHF From the Cell Line CHI
Triggers Both CTL and IgM Responses in an Antigen-Specific Manner

| Final Dilution ($\mu$g protein) CHI-ASHF Added to Culture | CTL Activity (% Lysis/culture) Stimulators: | | B Cell Activity• (AFC/Culture) | |
|---|---|---|---|---|
| | *B*2-FTA | *B*13-FTA | *B*2-CRBC | *B*13-CRBC |
| Without ASHF | <1 | <1 | 4±4 | 4±2 |
| 1/20 (42) | 34.6±6.0 | 5.3±1.7 | 92±8 | 44±11 |
| 1/60 | NT | NT | 316±24 | 156±41 |
| 1/200 (4.2) | 32.8±4.7 | <1 | 108±23 | 12±4 |
| 1/600 | NT | NT | 100±11 | 36±30 |
| 1/2,000 (0.42) | 18.7±4.4 | <1 | NT | NT |
| 1/20,000 | <1 | <1 | NT | NT |
| LPS | NT | NT | 276±30 | 472±39 |
| IL2 | 64.9±4.8 | 39.5±4.0 | NT | NT |

Antigen-affinity purified ASHF from the cell line CHI was tested for its ability to trigger CTL or AFC responses as described in Materials and Methods and previously (239,290). In the assay for help for CTLp, $10^5$ CBA thymocytes were cultured with irradiated *B*2-FTA- or *B*13-FTA-pulsed (BALB/c x CBA/J)F1 stimulators and graded doses of ASHF, IL2, or medium.

•Results provided by Dr. C. Shiozawa.

profile of CHI-ASHF from a DEAE-Sephacel anion-exchange column is illustrated in Figure 5. Although the specific ASHF activity is considerably increased by antigen-affinity chromatography (20- to 40-fold) (294) extraneous proteins are still present (as seen in Figure 5).

Since we have observed that ASHF preparations help a CTL response most efficiently if the stimulator cells are of the same H-2 haplotype as the CHI T*h* line (H-$2^k$)(29,239) all ASHF fractions were assayed for activity on CTLp in cultures containing BALB/c (H-$2^d$) responder thymocytes and *B*2-CRBC-pulsed CBA/J (H-$2^k$) adherent stimulator cells. In parallel, triggering activity on CBA/J B cells was tested. Table 12 demonstrates that ASHF-activity in the induction of CTL responses (ASHF-*CTL*) occurs exclusively in DEAE-Fraction E. In contrast, the ASHF activity that acts on B cells (ASHF-*B*) is contained in fraction C. The unfractionated CHI-ASHF from which the DEAE-fractions were derived, was highly active and antigen-specific in its ability to trigger both CTL and AFC responses. Preliminary experiments indicate that Fraction E does not suppress the induction of AFC by Fraction C and also indicates that fraction C contains more than one of the molecular species required in the triggering of AFC responses (C. Shiozawa, unpublished observations).

The data demonstrate the existence in CHI-ASHF of at least two functionally and physicochemically distinct ASHF that are both specific for *B*2-CRBC antigens. Residual antigen-nonspecific AFC-inductive activity is concentrated in fraction B. A more detailed analysis of the ability of DEAE-fractionated CHI-ASHF to stimulate B cell proliferation and differentiation has been reported previously (292).

### Preferential Triggering of CTL but not IgM Responses by ASHF Derived from Clone 4C6

Although CHI has been maintained *in vitro* for approximately two years, the cells of this line are phenotypically heterogeneous, as illustrated in Table 3 (chapter III). To minimize heterogeneity, CHI was cloned by limiting dilution. We isolated a clone, 4C6, able to help the induction of thymocyte CTLp in a "carrier-specific" manner in response to *B*2-CRBC

Figure 5

DEAE elution profile of CHI-ASHF

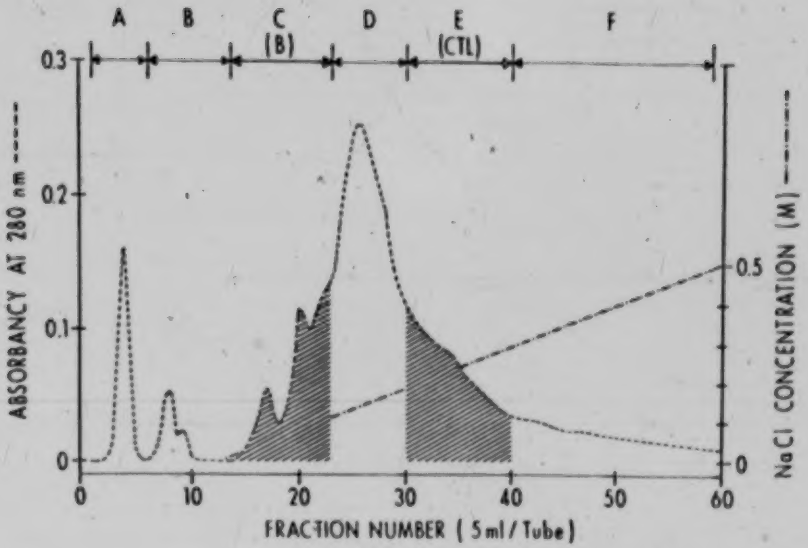


Figure 5: Protein elution profile of antigen-affinity purified supernatant of the cell line CHI, subjected to DEAE-Sephacel chromatography. Among the pools of fractions (A to F), ASHF*B* and ASHF*CTL* activities were recovered from fractions C and E, respectively (See Table 12).

TABLE 12

ASHF Activity for CTL Induction and for the Induction of B cell IgM Responses are Separable by Ion-Exchange Chromatography

| CHI-ASHF DEAE-Fraction | Protein µg/ml | CTL Activity (% Specific Lysis/Culture ± S.E.) |  | B Cell Activity[*] (AFC/Culture ± S.E.) |  |
|---|---|---|---|---|---|
|  |  | *B*2-FTA Stimulators | *B*13-FTA Stimulators | *B*2-CRBC | *B*13-CRBC |
| Without ASHF | — | <1 | <1 | 12±2 | 20±8 |
| A | 29 | <1 | <1 | 132±18 | 104±24 |
| B | 16 | <1 | <1 | 72±8 | 56±10 |
| C | 60 | <1 | <1 | 276±18 | 64±10 |
| D | 376 | <1 | <1 | 96±14 | 56±8 |
| E | 113 | 27.6±3.0 | <1 | 32±8 | 24±4 |
| F | 83 | 9.6±1.3 | 8.5±2.1 | 78±12 | 56±8 |
| LPS |  | NT | NT | 244±20 | 312±40 |
| IL2 |  | 27.3±4.5 | 21.4±1.4 | NT | NT |

The biological activities of ASHF-*CTL* and ASHF-*B* were recovered from distinct fractions after DEAE-Sephacel chromatography of antigen-affinity purified supernatant from the T*h* line CHI (see Fig. 5). A 1/60 final dilution of ASHF (1.0 µg protein-Fraction C) for B cell culture and a 1/180 final dilution (0.63 µg protein - Fraction E) for CTL culture are shown in this table. At no dilution tested (1/30 - 1/540) were the antigen-specific B cell- or CTL-inductive activities found in fractions other than C or E, respectively (not shown). In the assay for CTL activity, BALB/c thymocyte responders were cultured with *B*2- or *B*13-CRBC FTA-pulsed CBA stimulator cells. Cytotoxicity is expressed as % Specific Lysis of 5 x $10^4$ CBA targets.

[*] Results provided by Dr. C. Shiozawa.

FTA-pulsed stimulator cells.

Table 13 demonstrates that unfractionated ASHF from clone 4C6, or the DEAE-fraction "E" of 4C6-ASHF, are able to trigger CTLp in an antigen-specific manner. Fractions E of 4C6-ASHF contain cross-reactive or nonspecific factor(s) able to induce a small (10.5%) CTL response to *B*13-FTA-pulsed stimulators. However the CTL response to *B*2-FTA-pulsed stimulators was significantly greater (39.4%) ($p<0.0005$). The location of ASHF activity in DEAE-fraction "E" of both 4C6-ASHF and CHI-ASHF suggests that a similar molecular species in both ASHF preparations is responsible for the carrier-specific induction of CTLp. Fraction F appears to contain some antigen-nonspecific lymphokines that are able to trigger weakly CTLp. In contrast to the highly efficient triggering of CTLp by 4C6-ASHF (106% of control IL2-stimulated CTLp), even the highest concentrations tested of the DEAE fractions obtained from this sample (Table 11) were only weakly active in the triggering of AFC responses (maximum 21% of control LPS-stimulated IgM AFC), and this activity was not antigen-specific (C. Shiozawa, unpublished observations). Clone 4C6 appears to secrete some antigen-nonspecific B cell-directed lymphokines that participate in the induction of AFC precursors. At 3-fold lower concentrations of the 4C6 ASHF fraction, efficient triggering of CTLp was maintained but no induction of B cells was observed (not shown).

### $Ca^{++}$-sufficient ASHF from Clone 4C6 is Stringently Restricted to Syngeneic Adherent Cells

Experiments presented in Chapter III (Tables 9 & 10) demonstrated that syngeneic H-2-encoded products are required to reconstitute the functional activity of antigen-binding moities in incomplete ($Ca^{++}$-deficient) ASHF from the heterogeneous T*h* line CHI. The number and type of H-2-encoded subunits present on complete and incomplete forms of ASHF are not known. Purification of functional activity on antigen-affinity columns indicates that both contain antigen-binding moities, presumably T*h*AR. The phenotypic analysis of clone 4C6 (Table 3 - Chapter III), demonstrating very few $Ia^{+}$ cells, suggested that ASHF derived

## TABLE 13

The Inductive Activities of DEAE-Fractionated and Unfractionated ASHF, Derived from Clone 4C6, Indicate that ASHF-CTL and ASHF-B are Distinct Molecular Species

| | | CTL Activity (% Lysis/Culture) | | B Cell Activity* (AFC/Culture) | |
|---|---|---|---|---|---|
| 4C6-ASHF DEAE Fraction | Protein μg/ml | Stimulators Pulsed with: *B*2-FTA | *B*13-FTA | *B*2-CRBC | Fraction of LPS-Stimulated Control |
| Without ASHF | | 3.9±4.7 | <1 | 4±1 | 0.005 |
| A | 170 | 9.1±1.4 | 2.4±2.0 | 108±24 | 0.15 |
| B | 14 | 2.8±4.1 | <1 | 24±6 | 0.03 |
| C | 92 | <1 | <1 | 152±44 | 0.21 |
| D | 391 | 5.8±3.4 | 9.9±4.0 | 60±10 | 0.08 |
| E | 176 | 39.4±2.5† | 10.5±1.0 | 76±4 | 0.10 |
| F | 60 | 1.1±2.5 | <1 | 52±18 | 0.07 |
| Unfractionated | 800 | 57.3±4.0 | 9.4±3.8 | 148±2 | 0.20 |
| LPS | | NT | NT | 732±30 | 1.0 |
| IL2 | | 54.2±4.9 | 50.9±3.8 | NT | NT |

Affinity-purified ASHF was prepared from supernatants of clone 4C6, followed by ion-exchange chromatography, performed as described in Materials and Methods. The elution profile of 4C6-ASHF was nearly identical to that of CHI-ASHF (Fig. 5). Experimental conditions are as described for Table 10. No fraction of 4C6-ASHF specifically triggered AFC responses at any dilution tested (not shown).

*Results provided by Dr. C. Shiozawa.

†$p<0.0005$

from 4C6 might be similar to the "incomplete" form of ASHF. Table 14 demonstrates that 4C6 ASHF, prepared under $Ca^{++}$-sufficient conditions, is even more stringently restricted in its requirements for syngeneic adherent cells than the "incomplete" ($Ca^{++}$-deficient) CHI-derived ASHF.

The most permissive assay system, i.e. the system that detects complete and incomplete forms of ASHF, employs adherent stimulator cells expressing MHC-encoded antigens that are syngeneic to the CBA/J ($H$-$2^k$)-derived T*h* line CHI, as illustrated in Tables 9 and 10. Similarly, the use of *B*2-FTA-pulsed CBA/J stimulators is able to detect antigen-specific triggering of BALB/c thymocytes by complete or incomplete CHI-ASHF or by 4C6 ASHF (Table 14 - line 1). When responder and stimulator cells are allogeneic to the CHI-ASHF-producing cells only the complete form of CHI-ASHF but not incomplete CHI-ASHF or 4C6-ASHF is able to trigger thymocyte CTLp (line 2).

The use of B10.BR stimulator cells demonstrates that 4C6-ASHF is not functionally identical to incomplete ASHF derived from CHI (line 3). Significant CTL responses were generated by incomplete CHI-ASHF but not by 4C6 ASHF in response to *B*2-FTA-pulsed B10.BR stimulators. The response of CBA/J thymocytes to *B*2-FTA-pulsed stimulator cells that are allogeneic to the ASHF-producing T*h* provides further evidence that incomplete CHI-ASHF and 4C6-ASHF are different (lines 4 and 5). In these experiments, CBA/J CTLp are induced specifically in response to *B*2-FTA-pulsed BALB/c or B10 stimulators by either complete or incomplete CHI-ASHF but not by 4C6-ASHF. This suggests that syngeneic adherent cells from the responder thymocyte population are able to overcome the requirements of incomplete ($Ca^{++}$-deficient) CHI-ASHF, but not of 4C6-ASHF, for splenic adherent stimulator cells expressing H-2-encoded molecules that are syngeneic to the ASHF-producing cells.

The differences between 4C6-ASHF and the "incomplete" ASHF prepared by EGTA treatment and antigen-affinity chromatography of supernatants from the clonally heterogenous T*h* line CHI are not clear. The similar location of functionally active ASHF-*CTL* in

TABLE 14

The Function of ASHF From Clone 4C6 is Stringently Restricted to Syngeneic Adherent Cells

| Type of Thymocyte CTLp | Type of *B*2-FTA-pulsed Stimulator Cells | Percent of Control (IL2) Response | | |
|---|---|---|---|---|
| | | Complete ASHF | Incomplete ASHF | 4C6 ASHF |
| Allogeneic to ASHF | | | | |
| 1. BALB/c | CBA | ≥100 | ≥100 | ≥100 |
| 2. BALB/c | B10 | ≥100 | 0 | 0 |
| 3. BALB/c | B10.BR | — | 35* | 0 |
| Syngeneic to ASHF | | | | |
| 4. CBA | BALB/c | 74 | ≥100 | 0 |
| 5. CBA | B10 | 90 | ≥100 | 0 |

Three different types of CBA-derived ASHF were compared with regard to their requirement for syngeneic stimulator cells. Preparation of complete ($Ca^{++}$-sufficient) and incomplete ($Ca^{++}$-deficient) ASHF from the T*h* line CHI has been described previously (Chapter II and ref. 239). ASHF was purified from clone 4C6 (derived from the T*h* line CHI) in the presence of $Ca^{++}$. The ASHF-inducible CTL responses against target cells syngeneic to the stimulators are presented as the percent of IL2-inducible control responses. ASHF preparations were assayed at several dilutions (not shown) with consistent results. All experiments included a specificity control to confirm the lack of induction with *B*13-FTA-pulsed stimulator cells. The results of several experiments are summarized.

*An unusually high response to IL2 occurred in this group The absolute percent lysis was similar to the levels obtained in the groups presented in line 1.

DEAE-eluates from DEAE "E" fractions of CHI-ASHF and 4C6-ASHF indicates both have similar electronegative charges (Tables 12,13 and Fig. 5). Both incomplete ($Ca^{++}$-deficient) CHI-ASHF and 4C6-ASHF require syngeneic H-2-encoded products for functional activity. However, the stringent requirements of 4C6-ASHF (Table 14) demonstrate that it may be even less complete than $Ca^{++}$-deficient CHI-ASHF. It is not known if Fraction E of CHI-ASHF and the unfractionated 4C6-ASHF have the same subunit composition and the same stringent requirement for syngeneic H-2-encoded products provided by splenic adherent cells.

Incomplete and complete forms of ASHF may differ qualitatively, quantitatively, or in both respects in their requirements for syngeneic H-2-encoded molecules on adherent cells. These requirements may be dictated by the number and or type of ASHF "subunits" on the ASHF. Qualitative or quantitative differences in expression of the relevant H-2-encoded molecules on B10.BR splenic adherent cells or CBA/J thymocyte adherent cells (lines 3-5) (in comparison to CBA/J splenic adherent cells - line 1) may be involved in their inability to reconstitute the functional activity of 4C6-ASHF. Cullen *et al.* (332) have reported differential glycosylation of murine B cell and splenic adherent cell Ia$\alpha$ chains. Sialic acid residues on B-cell Ia$\alpha$ chains appear to prevent their ability to activate T cells (333). Studies by Janeway and his colleagues demonstrate that quantitative variation in Ia antigen expression plays an important role in T lymphocyte activation (334,335). Splenic and thymic adherent cells may differ quantitatively or qualitatively in the expression or post-translational modification of Ia molecules. Further functional and molecular characterization of these different forms of ASHF are required.

### D. Discussion

Large gaps exist in our understanding of how T*h* functions may selectively induce a particular class of immune response. Some evidence suggests the existence of subsets of T*h* that differ qulitatively in the class of immune response they are able to induce (336,337). Antigen-nonspecific lymphokines such as BCGF (65) BCDF$\gamma$ (66,67) and IL2 (52,338) appear to exhibit target specificity in the activation of B or T lymphocytes. Some studies indicate that ASHF act selectively on T or B cells (50,51,279,294,298). However, the comparison of the ability of the same ASHF preparations to induce CTL or antibody responses has never been previously reported.

In one of the earliest reports of antigen-specific cell-mediated cytotoxicity, Rosenau and Moon (339) were not able to detect antibodies against the target cells in animals that mounted a cell-mediated cytotoxic response. Although other interpretations of these results can be made, this situation may be analogous to the phenomena of "immune deviation" (64,340). The induction of a CTL response to virally-infected cells would offer an organism greater protection than would the induction of a class of antibody that does not fix complement, for example. Antibodies might also block the target sites available for recognition by CTL, NK, or DTH cells. In contrast, antibodies would be more effective in combatting viremia or septicemia. The mechanisms of immune class regulation are unclear although a theoretical model and data in support of it have been reported (64,81,341,342).

The results presented in this chapter and elsewhere (330) suggest that ASHF-*CTL* and ASHF-*B* constitute antigen-specific primary inductive signals that selectively regulate the class of immunity activated by antigen. The ASHF activities for CTL and B cell responses are demonstrated to be distinct and separable. Ion-exchange chromatography is able to resolve ASHF-*CTL* and ASHF-*B* into two separate peaks. Further support for our distinction between ASHF-*CTL* and ASHF-*B* is provided by the evidence that the bulk of ASHF secreted by clone 4C6 is ASHF-*CTL*. At present, our definition of ASHF-*CTL* is operational, in that although it is expected that ASHF-*CTL* is triggering CTLp directly, it is also possible that ASHF is triggering thymocyte T*h* precursors, which then trigger the CTLp. The functional differences of "incomplete" CHI-ASHF and 4C6-ASHF (Table 14) may reflect their preferential interaction with the precursors of T*h* or with CTLp.

It is clear that ASHF-*CTL* and ASHF-*B* are functionally disctinct. The molecular basis for these differences, however, is not. The studies of Shiozawa *et al* (294) demonstrate that ASHF-*B* consist of antigen-binding subunits and antigen-nonspecific subunits that area associated in the presence of $Ca^{++}$. Both types of subunits are required for functional activity. Functional analysis of effluents and eluates of ASHF purified on CRBC-affinity columns in the presence of EGTA demonstrated that both the effluent and the eluate fractions were able to synergize in the induction of B-cell proliferative responses or AFC activity. Further analysis of DEAE Fraction C of ASHF by G-100 gel filtration demonstrated the existence of two distinct peaks that synergized in the generation of AFC (C. Shiozawa, unpublished observations).

The assay system used to detect ASHF-*B* appears not to be "permissive" in its ability to detect incomplete forms of ASHF. Qualitative and/or quantitative insufficiencies of "completor" subunits or T*h* in this *in vitro* culture system may not permit detection of "incomplete" forms of ASHF-*B*, in contrast to the permissive ASHF-*CTL* assay system. Further experiments are necessary to determine if the requirements for "completor" subunits in the assays for ASHF-*B* and ASHF-*CTL* influence the apparent differences between these two forms of ASHF. The inability of Fraction E to suppress AFC responses induced by Fraction C indicates that this type of mechanism is not responsible for the inactivity of Fraction E in triggering AFC responses (C. Shiozawa, unpublished observations). The ability of unfractionated CHI-ASHF to induce both CTL and IgM responses (Table 11) also argues against this possibility. Studies by Lonai *et al.* (294) demonstrate that IA$\beta$ and IE$\alpha$ determinants are present on their ASHF-*B*. This ASHF, like the ASHF reported by Jaworski *et al.* (290) will not trigger H-2-incompatible B cells. The accidental or intentional dissociation of ASHF-*B* subunits may influence both its inductive activity and H-2-restriction properties, similar to the functional analysis of ASHF-*CTL* (Chap III, and ref. 29,239).

Table 14, demonstrating the stringent requirements of 4C6-ASHF for triggering CTLp (but not AFC precursors - Table 13), suggests that this form of ASHF is more incomplete than $Ca^{++}$-deficient CHI-ASHF. The 4C6-ASHF and the DEAE fraction E of CHI-ASHF may consist of "naked" T*h*AR. Antigen-nonspecific subunits may be required to make this moiety identical to the incomplete ($Ca^{++}$-deficient) CHI-ASHF. Additionally, H-2-encoded syngeneic adherent cell products are required to make $Ca^{++}$-deficient CHI-ASHF functionally complete and H-2-unrestricted at either the level of interactions with CTLp or with APC (Chap. III - Tables 9 & 10). In the permissive CTL induction system (using syngeneic splenic adherent cells) all necessary antigen-nonspecific subunits are provided by adherent cells to form a "complete" ASHF. The ability of unfractionated CHI-ASHF to trigger CTL responses renders in unlikely that suppressive moities present in fraction C are obscuring the effect(s) of ASHF-*CTL* present in this fraction. It is not clear why DEAE fraction C of CHI-ASHF is unable to trigger CTL responses.

As discussed previously in this thesis, a number of studies suggest that ASHF is composed of antigen-binding subunits, presumably T*h*AR, and subunits that do not bind antigen (Ia molecules). Data presented in the previous chapter and elswhere (29,239,294) indicate that both antigen-specific and antigen-nonspecific subunits are required to form a functionally active "complete" ASHF. Evidence presented in the following chapter strongly suggests that ASHF contain T*h*AR. It is reasonable to expect that T*h*AR units are antigenically monovalent. As discussed in Chapter I, Ia antigens may function as a polymorphic glue to make the antigenically-monovalent T*h*AR functionally multivalent. Multivalency may be required to achieve sufficiently avid binding to permit signal delivery to T or B lymphocytes by a tertiary complex of antigen, T*h*AR, and Ia antigens. Additionally, Ia molecules are suggested to serve as structures that interact with identical or nonidentical complementary molecules on B or T cells.

There are 2 possible explanations for our observation of distinct ASHF-*CTL* and ASHF-*B*: (a) there exist completely different ASHF isotypes, produced by two different subsets of class-specific T*h* or (b), a single type of T*h*-derived antigen-specific receptor is associated with different numbers and/or types of antigen-nonspecific subunits, derived from adherent cells, to form either ASHF-*CTL* or ASHF-*B*. The type of antigen-nonspecific subunit(s) contained in an ASHF complex may dictate its preferential interaction with T or B lymphocytes. Identification of the subunits of ASHF-*CTL* and ASHF-*B* will enable the direct testing of this hypothesis.

# V. FURTHER FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF ASHF-*CTL*.

## A. Introduction

Experiments presented in the previous chapters have characterized the antigen-specificity and H-2-restriction properties of *B2*-CRBC-specific ASHF from the T*h* line CHI and from clone 4C6. To confirm and extend these observations, experiments analyzing ASHF produced by other cloned T*h* lines that recognize CRBC antigens and by a T cell hybridoma that recognizes the synthetic polypetide Poly-18 (343,344) are presented in this chapter.

Criss-cross specificity experiments comfirm the antigen-specific induction of CTLp by ASHF, observed in the one-way specificity analyses presented previously (Chaps III, IV and Ref. 29,239,330). Analysis of the target cell specificities of ASHF-induced CTL confirm that ASHF is not a polyclonal activator.

It was important to determine if linkage of determinants recognized by ASHF and CTLp is required for the generation of CTL responses. As discussed previously (Chap I and Ref. 29), a requirement for linked recognition has been observed in the induction of antibody and DTH responses (10-20,28). Ther is no *a priori* reason to suggest that this requirement does not also occur in the induction of CTL responses. The ability of a requirement for linked recognition to minimize the induction of "bystander" CTLp provides a teleological argument for its existence. Also, evidence from some but not all experimental systems demonstrates this requirement in the induction of CTLp (245, 249-251).

Dekruyff *et al.* (291) have demonstrated a requirement for linked recognition in the induction of AFC responses by ASHF. This type of analysis of ASHF has not been reported previously in the induction of CTL responses. Experiments presented in this chapter demonstrate that determinants recognized by ASHF and CTLp must be present on the same stimulator cell for effective triggering of CTLp, thus demonstrating a requirement for at least intrastructurally linked (18) recognition in the induction of primary CTL responses. Once triggered by ASHF, CTLp are expected to express receptors for antigen-nonspecific lymphokines that mediate maturation and clonal expansion. It seems likely that in the absence of inhibitors, antigen-nonspecific lymphokines obscure the role(s) of ASHF-mediated events in CTLp induction occurring *in vitro*. We have avoided this problem through the use of ASHF as a source of help that does not include antigen-nonspecific lymphokines able to participate in CTLp induction.

The antigen-specific binding and functional activities of ASHF have suggested that they contain clonally-expressed T*h*AR. Depletion of functional ASHF activity by antibodies recognizing V*h*-framework determinants also support the validity of this assumption (288,298,310,311). However, formal proof that ASHF contain T*h*AR is lacking. Experiments presented in this chapter provide very strong evidence that ASHF contain determinants present on the $\beta$ chain of T*h*AR. Furthermore, these experiments demonstrate binding of T*h*AR to nominal antigen in the absence of MHC products. Experiments are in progress to isolate and identify other subunits of ASHF-*CTL*.

The accumulation of evidence presented in this thesis provides strong support for the previously described model (p.35) of CTLp induction by soluble forms of T cell receptors. It is suggested that ASHF constitute primary signals for the induction of T or B lymphocytes.

## B. Materials and Methods

*Antigens.* The preparation of CRBC-FTA has been described (Chap. II and Ref. 239). The synthetic polypeptide Poly-18 (343) was prepared by Dr. E. Fraga in the laboratory of Dr. B. Singh as described (344). The repetitive amino acid sequence in this polymer is Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$. Gel filtration of Poly-18 was performed by Dr. E. Fraga to obtain a polymer with an average molecular weight of 11 Kd for experiments described in these studies.

*Th Lines and Hybridomas.* The cloned CRBC-reactive T*h* lines LB2-1, LB19-1 and GK15-1 were developed by Dr. Tim Mosmann (DNAX Research Institute - Palo Alto, CA) using the methods of Kimoto and Fathman (322). Lines LB2-1 and LB19-1 are of C57Bl10/J (H-$2^{b}$) origin. Line GK15-1 is of CBA/J (H-$2^{k}$) origin. All three lines recognize relatively nonpolymorphic determinants on CRBC. The T*h* lines LB2-1 and GK15-1 release IL2 and IL3 in response to H-2 syngeneic APC plus all MHC haplotypes of CRBC except *B*13. Line LB19-1 recognizes determinants common to all MHC haplotypes of CRBC tested, including *B*13. None of these T*h* lines recognize determinants present on SRBC (T. Mosmann, personal communication). The T*h* clones MD13-5.1 and MD13-10 were developed by Dr. M. Giedlin in the laboratory of Dr. T. Mosmann.

A T*h* line that recognized Poly-18 was derived from BALB/c mice by Dr. A. Fotedar using the methods of Kimoto and Fathman (322). Cells from this line were then fused with the AKR-derived tumor partner BW5147 (345). HAT-resistant hybrids were secreened for their ability to produce IL2 specifically in response to APC plus Poly-18. Experiments using inhibition of IL2-release by monoclonal antibodies recognizing $IA^{d}$ determinants indicate that the T cell hybridoma Poly-18.68 recognizes nominal antigen in association with these products (B. Singh, personal communication).

*Preparation of Antigen-Specific Helper Factors.* For the purification of ASHF from supernatants of the T*h* lines LB2-1, LB19-1, and GK15-1, antigen-affinity columns consisting of *B*2-CRBC coupled to Sepharose 4B were used, as described previously (290). Supernatants from Poly-18.68, prepared by J. Widtman, were purified on Poly-18-Sepharose columns by Dr. B. Singh. Antigen-bound fractions were eluted with 2M NaCl, dialyzed exhaustively against PBS, and concentrated.

*Purification of KJ16-133 Monoclonal Antibody.* The monoclonal antibody KJ16-133 that recognizes determinants on the $\beta$ chain of T cell antigen receptors (158) was provided by Drs. P. Marrack and J. Kappler of the National Jewish Hospital in Denver, Colorado. A two step purification was employed to enrich the concentration of this rat immunoglobulin. Crude culture supernatant was dialyzed against 0.01M $K_2HPO_4$/0.15 M NaCl (pH 7.25). This sample was applied to a CM Affi-Gel Blue column (Biorad - Richmond, CA) to deplete albumin. The effluent was collected, concentrated, dialyzed, and applied to a DEAE-Sephacel anion-exchange column (Pharmacia, Uppsala, Sweden). Fractions eluted with 0.025M. 0.08M, or 0.3M phosphate buffer were pooled, concentrated, dialyzed, and coated to ELISA plates for analysis of the presence of rat immunoglobulins by ELISA, as described subsequently. Only the 0.025M-eluate contained significant reactivity, suggesting that KJ16-133 is an IgG subclass (not shown).

*ELISA for Detection of β chains.* This assay was based on a previously developed procedure for the detection of rat immunoglobulins (346). One hundred μl of dilutions of antigen-affinity-purified preparations in a carbonate coating buffer (pH 9.6) were added to the wells of Dynatech Immulon II ELISA plates (Dynatech - Alexandria, VA) and incubated for two hours at 37°C or overnight at 4°C. After washing the plates 5X in double-distilled water containing 0.25% Tween 20 (Sigma - St, Louis, MO), 100 μl of a 1/4 or 1/8 dilution of the CM-Affigel- and DEAE-purified KJ16-133 was added to each well. Due to the extremely limited supply of KJ16-133 available, only single wells were tested at each dilution of supernatant or ASHF. The plate was incubated for 1 hr at 37°C and subsequently washed 5X. One hundred μl of a 1/400 dilution of a peroxidase-labelled rabbit anti-rat IgG (Heavy and Light Chains) (Cappel - West Chester, PA) were added to each well prior to incubation at 37°C for 1 hr. After washing 5X, 100 μl of $H_2O_2$ and 2,2-Azinodi-(3-Ethylbenzthiazoline Sulfonic Acid) (Sigma - St. Louis, MO) in Citric Buffer were added to each well. After 30 minutes at room temperature, the O.D. at 414 nm was quantitated on a Titertek Multiscan microplate reader (Flow Laboratories - McLean, VA).

*Indirect Immunofluorescent Analysis.* Procedures for indirect immunofluorescent analysis with KJ16-133 have been described by Haskins *et al.* (158). A modified version was used in these experiments. Briefly, 5 x $10^5$ - 1 x $10^6$ cells were suspended in 100 μl of diluent (PBS, 2% FCS, 0.2% sodium azide) or in diluent containing a 1/4 or a 1/10 dilution of purified

KJ16-133. Samples were incubated for 30 minutes at 37°C. Subsequently cells were centrifuged at 500 x G for 5 minutes. As the fluorescein-coupled mouse-anti-rat kappa antibody used by Haskins *et al.* (158) was not commercially available, a flouresceinated rabbit-anti-rat Ig (Boehringer Mannheim - Indianapolis, IN) was substituted. Fifty μl of a 1/30 dilution of this fluoresceinated second reagent which had been previously centrifuged at 50,000 x G for 15 minutes to remove aggregated immunoglobulins was added to all wells prior to incubation for 30 minutes at 37°C. Cells were washed three times with diluent and resuspended in 25 μl of diluent. At least 200 cells were counted for each sample. Any cell clumps or the few dead cells in each preparation were excluded.

For analysis of Lyt1 and Lyt2 antigens, the TIB 104 and TIB 105 rat monoclonal antibodies (345), from hybridomas purchased from the American Type Culture Collection (Rockville, MD) were used as ascites or as culture supernatants at an optimal concentration. An anti-Thy 1.2 monoclonal antibody (New England Nuclear - Lachine, Quebec) was used in these studies at a 1/500 dilution in combination with a fluoresceinated goat-anti-mouse IgM (Cappel - West chester, PA). With these antibodies, the first step and second steps of labelling were performed at 4°C for 1 hour each.

*Preparation of KJ16-133 Affinity Column.* Cyanogen bromide activated Separose 4B (Pharmacia Uppsala, Sweden) was swollen for 20 minutes in 1 mM HCl. The gel was washed with coupling buffer (0.2M $NaHCO_3$/ 0.5 M NaCl - pH 8.5) and mixed with the semi-purified KJ16-133 antibody (1.85 mg protein) in coupling buffer. After being mixed for 12 hr. at 4°C the gel was washed in coupling buffer containing 0.2 M glycine (Bethesda Research Laboratories - Gaithersburg, MD), resuspended in the same, mixed for 12 hr. at 4°C, and washed with 2M NaCl followed by PBS. The effficiency of coupling the protein to the gel was 63%.

## C. RESULTS

### Specificity Analysis of LB19-1 and Poly18.68 ASHF

The adaptability of the CTLp induction assay developed in these studies enabled functional analysis of T*h* and ASHF that recognize a wide variety of antigens. Table 15 demonstrates that ASHF from LB19-1 triggers CTLp when the (BALB/c x B10)F1 stimulators present *B*2-CRBC antigens but not when they are exposed to the synthetic polypeptide Poly-18. Other experiments have demonstrated that LB19-1 ASHF is able to trigger significant CTL responses when stimulator cells are pulsed with *B*14 FTA (unpublished observations). This is consistent with the ability of LB19-1 cells to proliferate, and release lymphokines (IL2 and IL3) in response to H-$2^b$ APC and *B*2 or *B*14 CRBC (T. Mosmann, personal communication). The LB19-1 T*h* cells have not been available for testing of their ability to trigger CTLp. It is presumed that they function in a manner similar to the L61 and CHI cells described previously (Chap. III - Tables 1 and 2).

Table 15 also demonstrates that in the same experiment, ASHF from the T cell hybridoma Poly 18.68 is able to trigger CTLp when the stimulator cells are pulsed with Poly 18 (1 μg) but not when they are pulsed with *B*2-FTA. Other experiments have demonstrated a criss-cross specificity in the triggering of CTL responses by the CRBC-specific GK15-1 ASHF (See Materials and Methods) and Poly-18.68 ASHF (unpublished observations). Similar criss-cross specificity analyses have utilized this system developed by Dr. Pilarski and myself to demonstrate the "carrier"-specific induction of CTLp by PPD- and KLH-reactive ASHF (D. Kilburn, personal communication). It is interesting that although Poly-18.68 ASHF efficiently triggers CTLp in an antigen-specific manner, as many as $10^4$ of irradiated (1500 rad) Poly 18.68 hybridoma cells are unable to trigger CTL responses under any conditions tested (unpublished observations). A number of mechanisms may be responsible for this inability to trigger CTLp. For example, secretion of ASHF by Poly 18.68 may be inhibited by irradiation. An alternative explanation is that this ASHF-producing hybridoma may also secrete

TABLE 15

Criss-cross specificity analysis of LB19-1 and Poly 18.68 ASHF

| | | | % Specific Lysis of B10 Targets (BALB/c x B10)F1 Stimulators Pulsed with: | |
|---|---|---|---|---|
| Dilution of LB19-1 ASHF | Dilution of Poly-18.68 ASHF | IL2 | *B2*-FTA | Poly-18 |
| — | — | — | 4.1±2.7 | <1 |
| — | — | + | 24.2±2.7 | 16.6±3.3 |
| 1/80 | — | — | 24.9±2.1 | 8.2±2.4 |
| 1/160 | — | — | 29.0±2.2 | 5.2±1.3 |
| 1/320 | — | — | 19.3±1.9 | <1 |
| 1/640 | — | — | 2.8±1.0 | <1 |
| — | — | — | 3.5±0.8 | 2.6±0.5 |
| — | — | + | 21.6±4.5 | 20.8±5.2 |
| — | 1/160 | — | 2.4±0.6 | 29.4±7.2 |
| — | 1/640 | — | 2.1±0.7 | 19.6±3.4 |
| — | 1/1280 | — | 3.0±0.9 | 18.4±5.5 |
| — | 1/2560 | — | 3.4±1.3 | <1 |

One x $10^5$ BALB.c thymocytes were cultured with the indicated dilutions of LB19-1 ASHF or Poly-18.68 ASHF and the irradiated (1500 rad) plastic-adherent fraction from 3 x $10^5$ (BALB/c x B10)F1 stimulators that had been pulsed with *B2*-FTA ($10^6$ CRBC equivalent) or Poly 18 (1 $\mu$g). Cytotoxicity was assayed after 5 days against C57Bl10/J (B10) Con A blast targets. Spontaneous/Detergent release of $^{51}$Cr from B10 blasts = 0.23.

immunosuppressive molecules that inhibit the induction of CTL responses. Antigen-affinity chromatography is necessary to detect ASHF produced by Poly 18.68.

### Target Specificity of ASHF-induced CTL

Analysis of the antigen-specificity of the CTL induced by ASHF indicated that ASHF is not a polyclonal activator (Chap III - Table 7). The alloantigens recognized by clonally-expressed CTR must be present in culture during ASHF-mediated induction. In order to further characterize this specificity, CTL activity by BALB/c thymocyte responders cultured with Poly-18-pulsed (BALB/c x CBA/J)F1 stimulator cells and Poly-18.68 ASHF or IL2 was examined. The results of these experiments are presented in Table 16.

The inability of the CTL generated in these cultures (Table 16) to lyse B10 targets (B10 antigens not present in culture) provides confirmation (see Table 7 legend) that ASHF is not a polyclonal activator. The most vigorous CTL responses were observed against CBA/J targets, that are syngeneic with the alloantigens encountered by BALB/c thymocytes during *in vitro* culture. In this particular experiment, IL2 induced greater CTL activity than did Poly-18.68 ASHF. The CTL generated in these cultures are also able to lyse C3H.A targets, that are identical with CBA/J in the K through IE regions of H-2. In contrast, CTL induced by ASHF or IL2 are capable of only weak lysis of C3H.OH targets that share only $D^k$-products with CBA/J. The reason for the preferential killing of targets expressing alloantigens encoded by the K end regions of H-2 are unclear but has been observed previously for thymocyte responders (L. Pilarski, personal communication). Table 16 demonstrates that anti-self (BALB/c) CTL were not induced by ASHF or IL2. Table 15 demonstrates that BALB/c thymocytes cultured with $H\text{-}2^b$ -expressing stimulator cells and IL2 or ASHF are capable of generating vigorous CTL responses that lyse $H\text{-}2^b$ targets.

## TABLE 16

### Target specificity of ASHF-induced CTL

| Poly 18.68 ASHF | IL2 | % Lysis of Targets | | | | |
|---|---|---|---|---|---|---|
| | | CBA/J | B10 | C3H.A | C3H.OH | BALB/c |
| — | — | 2.0±0.8 | 4.5±4.3 | 2.2±1.3 | <1 | <1 |
| — | + | 41.6±3.2 | 5.0±2.6 | 25.6±3.4 | 12.1±1.1 | 2.2±1.0 |
| + | — | 27.2±5.1 | 6.2±3.6 | 16.9±2.9 | 7.4±2.1 | <1 |

One x $10^5$ BALB/c thymocytes were cultured with the irradiated adherent fraction from 3 X $10^5$ (BALB x CBA/J)F1 stimulator cells that had been pulsed with 1 μg of Poly-18. Medium, IL2, or an optimal (1/200 - see Table 15) dilution of Poly-18.68 ASFH were added to each well. Cytotoxicity was assayed after 5 days against Con A blast targets from the mouse strains indicated. Spontaneous/detergent release of $^{51}Cr$ from target cells - CBA/J=0.18; B10=0.16; C3H.A=0.28; C3H.OH=0.33; BALB/c=0.19. No CTL responses were generated in cultures containing BALB/c thymocytes, Poly-18.68 ASHF, and unpulsed stimulators (not shown).

## CTL Induction Requires Intrastructural Linkage of Determinants Recognized by ASHF and CTLp

As discussed at length in Chapter I, evidence in some experimental systems suggests a requirement for physical association of the determinants recognized by T*h* and CTLp in the triggering of CTL responses (245,249-251). To determine whether or not ASHF exhibited a requirement for linked or cognate interactions in the in the triggering of CTLp, it was essential to provide the operationally-defined "hapten-carrier" in both linked and unlinked forms. A linked form of antigen is used in experiments presented in Table 15. The (BALB/c x B10)F1 adherent stimulators express the H-$2^b$ alloantigens (haptens) recognized as foreign by clonally-expressed receptors on BALB/c CTLp. This same population of stimulator cells present *B*2-CRBC antigens or Poly-18 (carrier determinants) for recognition by LB19-1- or Poly-18.68-ASHF, respectively. As the "hapten" and "carrier" determinants are on physically distinct molecules in this system, the linkage between them on the cell-surface is intrastructural rather than intramolecular, as defined by Lake and Mitchison (18).

Experiments using the operationally defined linked and unlinked forms of hapten and carrier are presented in Table 17. By exposing separately stimulator cells of each parental type (BALB/c or B10) to *B*2-FTA or Poly-18, linked and unlinked forms of antigen are able to be compared. In order to saisfy our criteria for linked recognition, both "hapten" and "carrier" determinants must be present in culture and both must be associated on the surface of the same stimulator cell population for induction to occur. Table 17 demonstrates that the CRBC-specific LB19-1 ASHF triggers effectively anti-H-$2^b$ CTLp in response to *B*2-pulsed B10 stimulators and Poly-18-pulsed BALB/c stimulators, that is when the hapten and carrier are on the same cells. In contrast, when both hapten and carrier are present in the same culture but in an unlinked form, that is on different stimulator cell populations (Poly-18-pulsed B10 and *B*2-FTA-pulsed BALB/c stimulators), only negligible CTL responses are generated by LB19-1 ASHF. Similar levels of cytotoxicity are induced by IL2 in response to either the linked or unlinked forms on antigen.

TABLE 17

Cytotoxic T lymphocyte induction requires intrastructural linkage of determinants recognized by LB-19 ASHF and CTLp

| | | | % Lysis of B10 Targets | |
|---|---|---|---|---|
| | | | Stimulators: | |
| Dilution of LB19-1 ASHF | Dilution of Poly-18.68 ASHF | IL2 | B10-*B2*-FTA<br>BALB/c-Poly-18 | B10-Poly-18<br>BALB/c-*B2*-FTA |
| — | — | — | <1 | 1.7±1.0 |
| — | — | + | 31.8±5.8 | 21.2±2.9 |
| 1/80 | — | — | 31.3±1.3 | 5.0±1.6 |
| 1/160 | — | — | 30.0±3.0 | 5.4±1.3 |
| 1/320 | — | — | 21.0±2.6 | 3.6±1.0 |
| 1/640 | — | — | 6.0±1.2 | 2.5±0.8 |
| — | — | — | 2.8±0.8 | 3.7±1.3 |
| — | — | + | 38.7±4.2 | 34.1±3.8 |
| — | 1/160 | — | 4.5±0.8 | 3.7±1.3 |
| — | 1/640 | — | 6.4±1.3 | <1 |
| — | 1/1280 | — | 3.8±1.8 | <1 |
| — | 1/2560 | — | 3.7±1.9 | <1 |

BALB/c or C57Bl10/J (B10) stimulator cells were pulsed with either *B2*-FTA or poly 18 (1 μg/ml) for 90 min. at 37°C. Subsequently, unbound antigen was removed by washing the cells twice. Antigen-pulsed stimulator cells ($1.5 \times 10^5$ of each type indicated) were added to each microwell. After two hours incubation at 37°C, non-adherent cells were removed. BALB/c thymocytes, ASHF and IL2 or medium were added as described in the legend to Table 15. Data presented in this table and Table 15 are from the same experiment. Poly-18.68 ASHF very efficiently helped CTL responses to Poly-18-pulsed but not to unpulsed F1 stimulators (Table 15).

The results presented in Table 17 could also be interpreted to indicate a requirement for H-$2^b$ -restricted recognition of nominal antigen (*B*2-FTA) by the LB19-1 (H-$2^b$ ) ASHF. As the BALB/c thymocyte responders are allogeneic to the LB19-1 ASHF-producing T*h*, the inability of LB19-1 ASHF to trigger CTL responses to Poly-18-pulsed B10 stimulators and *B*2-FTA-pulsed BALB/c stimulators could result from a lack of association between H-$2^b$ -encoded products and *B*2-FTA. Our experimental evidence indicates that this is not likely. LB19-1 ASHF binds to *B*2-CRBC in the absence of H-2-encoded products. Furthermore, Table 18 demonstrates that LB19-1 ASHF exhibits a requirement for linked recognition when the haplotypes of both stimulator cell populations and the responder thymocytes are allogeneic to LB19-1. In this experiment, LB19-1 ASHF is able to trigger CBA/J CTLp in response to *B*2-FTA pulsed BALB/c stimulators and unpulsed CBA/J adherent cells but not in response to *B*2-FTA pulsed CBA/J stimulators and unpulsed BALB/c stimulators. In the former, but not in the latter, the hapten (H-$2^d$ alloantigens) and carrier (*B*2-FTA) are intrastructurally linked. In neither combination are the carrier determinants presented in the context of H-$2^b$ -encoded products. This experiment demonstrates that LB19-1 ASHF requires linkage of hapten and carrier for effective triggering of CTLp but is not H-2 restricted in its requirement for nominal antigen. Furthermore, the ability of LB19-1 ASHF to trigger CTL responses when both responder and stimulator cells are allogeneic to the ASHF-producing LB19-1 T*h* indicates that this ASHF is functionally "complete" (see Chaps III & IV).

The results presented in Table 19 demonstrate that the requirement for linked recogntion by ASHF from the T*h* line GK15-1 is not due to H-2-restricted recognition of nominal antigen (*B*2-FTA). Like LB19-1 ASHF, GK15-1 ASHF require intrastructural linkage of determinants recognized by ASHF and CTLp. Nearly identical levels of CTL responses to linked or unlinked forms of antigen were induced by IL2 and this does not require the presence of *B*2-FTA, in contrast to GK15-1 ASHF. In this experiment the CBA/J-derived GK15-1 ASHF is able to induce H-$2^d$ -reactive CBA/J thymocyte CTLp in response to

TABLE 18

The requirement for linked recognition by LB19-1 ASHF is not due to H-2-restricted recognition of nominal antigen.

| Dilution of LB19-1 ASHF | IL2 | % Lysis of BALB/c Targets<br>Stimulator Cells:<br>BALB/c-*B*2-FTA<br>CBA/J-unpulsed | <br><br>CBA/J-*B*2-FTA<br>BALB/c-Unpulsed |
|---|---|---|---|
| — | — | 5.0±2.0 | 3.3±1.6 |
| — | + | 40.1±6.4 | 35.2±3.6 |
| 1/100 | — | 17.9±3.7 | 3.4±1.9 |
| 1/200 | — | 12.1±3.2 | 2.4±2.0 |
| 1/400 | — | <1 | <1 |

BALB/c or CBA/J stimulator cells were either pulsed with *B*2-FTA ($10^6$ CRB C equivalent) or were not exposed to this carrier (unpulsed). The indicated populations of irradiated (1500 rad) adherent stimulator cells were cultured with 2 x $10^5$ CBA/J thymocytes and dilutions of LB19-1 ASHF or IL2 in 0.2 ml. Cytotoxicity against BALB/c Con A targets was assayed after 5 days. Spontaneous/detergent release of $^{51}$Cr from targets = 0.20. The carrier-specificity of LB19-1 ASHF is demonstrated in Table 17.

TABLE 19

Cytotoxic T lymphocyte induction by GK15-1 ASHF requires linkage of determinants recognized by ASHF and CTLp.

| Dilution of GK15-1 ASHF | IL2 | % Lysis of BALB/c Targets | | | |
|---|---|---|---|---|---|
| | | Stimulators: | | | |
| | | F1 + *B*2 | F1 Unpulsed | BALB/c-*B2*FTA CBA-Unpulsed | CBA-*B*2FTA BALB/c-Unpulsed |
| — | — | 3.5±2.0 | 3.1±1.5 | 1.6±3.3 | <1 |
| — | + | 37.7±1.9 | 37.5±5.9 | 37.5±1.2 | 36.3±5.0 |
| 1/100 | — | 31.2±6.3 | 4.4±2.2 | 36.4±4.5 | 4.3±1.6 |
| 1/200 | — | 22.2±3.5 | <1 | 26.3±1.7 | 5.0±1.5 |
| 1/400 | — | 5.7±1.7 | <1 | 11.2±3.0 | 5.4±3.5 |

BALB/c, CBA/J or (BALB/c x CBA/J) F1 stimulators were pulsed with MEM containing *B*2-FTA or MEM without FTA (unpulsed stimulators) for 90 minutes at 37°C. After washing unbound antigen, either 3X$10^5$ F1 or 1.5 X $10^5$ of each parental type stimulator were added to each well as indicated. Non-adherent cells were washed out prior to the irradiation (1500 rad) of stimulator cells. Medium, the indicated dilutions of ASHF, or IL2 and $10^5$ CBA/J thymocytes were added.

*B*2-FTA pulsed BALB/c stimulators and unpulsed CBA/J adherent cells but not in response to *B*2-FTA-pulsed CBA/J stimulator cells and unpulsed BALB/c stimulator cells. GK15-1 ASHF thus requires association of hapten and carrier but does not require association of H-2$^{k}$ -encoded molecules and *B*2-FTA for triggering of CTLp

Table 17 demonstrates that the pattern of responses induced by Poly-18.68 ASHF is different from that of LB19-1 and GK15-1 ASHF. In contrast to the specific induction of CTL responses to Poly-18-pulsed (BALB/c x B10)F1 stimulators (Table 15), Poly-18.68 ASHF is unable to induce significant BALB/c CTL responses to either Poly-18-pulsed BALB/c stimulators and *B*2-FTA-pulsed B10 stimulators or to Poly-18-pulsed B10 stimulators and *B*2-FTA-pulsed BALB/c stimulators. It has been observed previously that H-2$^{b}$ mice are nonresponders to Poly-18 (343). The inability of Poly-18 to effectively associate with H-2$^{b}$ -encoded products may explain the absence of a response to Poly-18-pulsed B10 stimulators and *B*2-FTA-pulsed BALB/c stimulators. The absence of intrastructural linkage between H-2$^{b}$ -encoded alloantigens and Poly-18 is the most likely explanation for the lack of Poly-18.68 ASHF-induced responses to *B*2-FTA-pulsed B10 stimulators and Poly-18-pulsed BALB/c stimulators. In addition, the Poly-18.68 ASHF may require recognition of nominal antigen in the context of H-2-encoded products for effective triggering of CTLp, unlike the ASHF derived from the T*h* lines LB19-1 and GK15-1 ASHF. Thus, either or both of these requirements for linked and H-2-restricted interactions may be operational in the induction of CTLp by ASHF.

### Antigen-Affinity Purified ASHF from Poly 18.68 and LB19-1 Contain $\beta$ Chains of T Cell Antigen Receptors

Comparisons of secretory and membrane forms of $\mu$, $\delta$, and $\gamma$ immunoglobulin heavy chains (274-277) suggests by analogy, that secretory forms of T*h*AR may also use many of the same C region exons as its membrane-bound counterpart. The report of a monoclonal antibody recognizing a C region allotypic marker (158) suggested, therefore, that this antibody might be useful in the detection of soluble forms of T*h*AR.

Initially we wished to determine if any of the T cell lines and hybrids currently in use expressed determinants recognized by KJ16-133. We were unable to detect any positive cells in the analysis of normal spleen cells, thymocytes, or a variety of T cell lines and hybrids using crude hybridoma culture supernatant and the procedures described in Materials and Methods. However, after purification of the crude supernatant (see Materials and Methods) we were able to label percentages of normal T cell populations similar to those reported by Haskins *et al.* (158). The results of indirect immunoflourescent analysis with KJ16-133 and other antibodies are listed in Table 20. Nylon-wool-purified spleen cells from BALB/c mice demonstrated 18.5% KJ16-133$^+$ cells (Line 1). Nylon-wool passed spleen cells from CBA/J mice demonstrated a similar percentage of positive cells (not shown). BALB/c and B10 thymocytes demonstrated 15.7% and 9.7% KJ16-133$^+$ cells, respectively (lines 2 and 3). These values are similar to those reported by Haskins *et al.* (156). The ASHF-producing hybridoma Poly 18.68 exhibited only 7.4% KJ16-133$^+$ cells (line 4). On other occasions, this value varied between 5 and 15% (not shown). To enrich the population of KJ16-133$^+$ cells the Poly 18.68 was sorted by FACS. Only the brightest 5% were retained. After 10 days culture of the sorted P18.68, indirect immunofluorescence demonstrated significant enrichment of KJ16-133$^+$ hybrids (Line 5). KJ16-133$^-$ contaminants in the KJ16-133$^+$ sorted population may explain the inability to attain a 100% KJ16-133$^+$ population of Poly-18.68 and further sorting may be required. An independently-derived hybridoma (Poly 18.12), that proliferates specifically in response to Poly-18 and APC (A. Fotedar, personal communication), on repeated testing lacked determinants recognized by KJ16-133 (Line 6).

Phenotypic analysis of the uncloned T cell lines, D*CP* and CPPD demonstrated 13.0% and 17.8% KJ16-133$^+$ cells, respectively (lines 7 & 8). Recent analysis of CPPD on two occasions demonstrated that this cell line had undergone a phenotypic shift and now 100% of CPPD cells are positive (line 9). Clones were derived from this cell line and are currently being tested for specificity in proliferation and in their ability to help CTLp. MD13-5.1 is a

## TABLE 20

### Indirect Immunofluorescent Analysis

| | | % Fluorescent Cells | | | |
|---|---|---|---|---|---|
| | Cell Type | KJ16-133 | Thy1.2 | Lyt1 | Lyt2 |
| 1. | BALB/c<br>Nylon-Wool<br>Purified Spleen Cells | 18.5 | 92.0 | 63.3 | 31.7 |
| 2. | BALB/c Thymocytes | 15.7 | — | 92.0 | 80.0 |
| 3. | B10 Thymocytes | 9.7 | — | 60.0 | — |
| 4. | Poly-18.68 | 7.4 | 100 | — | — |
| 5. | Poly-18.68 (Sorted) | 39.4 | 100 | — | — |
| 6. | Poly-18.12 | 0 | 100 | — | — |
| 7. | *D*CP | 13.0 | — | 10.3 | — |
| 8. | CPPD (August '84) | 17.8 | — | 33.3 | — |
| 9. | CPPD (October '84) | 100 | — | — | — |
| 10. | MD13-5.1 | 0 | 100 | — | — |
| 11. | MD13-10 | 100 | 100 | — | — |

Procedures for immunofluorescence are described in Materials and Methods. Poly-18.68 (Line 4) is an ASHF-producing hybridoma (See Table 15). Line 5 describes the phenotype of KJ16-133 positive cells sorted by flow cytometry from the previous population. Poly-18-12 (line 6) is a hybridoma, generated independently of Poly-18.68, that secretes IL2 in response to Poly-18. *D*CP and CPPD (Lines 7 - 9) are described in Chapters II and III. MD13-5.1 and MD13-10 are BALB/c T*h* lines developed by Dr. M. Giedlin in the laboratory of Dr. T. Mosmann (DNAX-Palo Alto, CA) that recognize determinants on *B*13 CRBC.

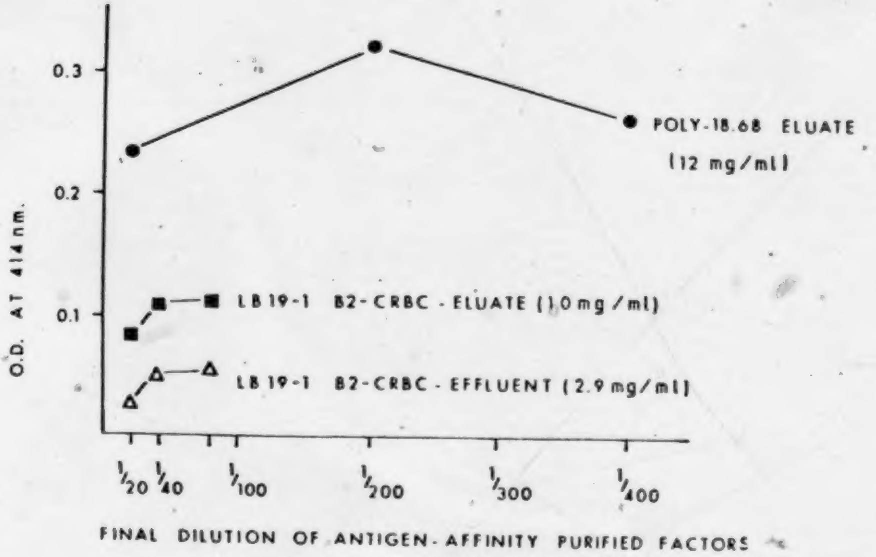
subcloned BALB/c T cell line that responds to *B*2, 13, 14, 15, and 19 CRBC but not to *B*21 (Line 10). It has no detectable Mls reactivity. MD13-10 is a cloned BALB/c T cell line with the same CRBC ractivity pattern as MD13-5.1. It also responds very strongly to Mls haplotypes a and d. Aproximately 100% of MD13-10 T cells express the KJ16-133 determinant (line 11). Analysis of more cloned T cell lines and hybrids will determine if there is a significant correlation between KJ16-133 positivity and Mls reactivity.

We wished to determine if our ASHF preparations contained $\beta$ chains of T*h*AR. Figure 6 demonstrates that the dialyzed 2M NaCl eluate containing molecules in Poly-18.68 SN that bound to a a Poly-18-Sepharose column exhibited significant reactivity with KJ16-133, as determined by ELISA. The eluate from LB19-1 supernatants chromatographed on a *B*2-CRBC affinity column also contained significant reactivity with KJ16-133 in the ELISA. In contrast, the effluents that did not bind to CRBC and therefore passed through the column in the effluent contained very little reactivity with KJ16-133. It is evident that the protein concentration of the samples did not correlate with reactivity with KJ16-133, suggesting that this assay is specific in its ability to detect $\beta$ chains of T cell receptors in ASHF preparations. Specific recognition of T cell receptor $\beta$ chains by KJ16-133 has been characterized previously (158).

Table 21 provides further confirmation of the reactivity of KJ16-133 with ASHF preparations. A new batch of Poly-18.68 ASHF also showed reactivity with KJ16-133 in the ELISA. Antigen-affinity purified Poly-18.68 ASHF prepared from serum-free culture supernatants also showed significant reactivity with KJ16-133, indicating that BSA or other components of FCS are not responsible for the reactivity with KJ16-133. Crude supernatants from the KJ16-133$^+$ T*h* line CPPD also demonstrated significant reactivity with KJ16-133. At no dilution of ASHF or CPPD SN was an O.D. of more than 0.056 obtained when PBS was substituted for KJ16-133 (not shown), demonstrating that the peroxidase-labelled rabbit anti-rat Ig is not directly recognizing ASHF.

Figure 6

Antigen-affinity purified ASHF from LB19-1 and Poly-18.68 contain $\beta$ chains of T cell receptors.



A 1/4 dilution of purified KJ16-133 was added to wells coated with the indicated dilutions of the effluents or 2M Nacl eluates from antigen-afffinity columns. The effluent from the Poly 18.68 supernatants was not available for testing. Protein concentrations were determined by absorbancy at 280 nm using BSA as a standard. Color development was undetectable in wells that were not coated with effluents or eluates (not shown). The antigen-specific CTL-inductive activity of the Poly 18.68 and LB19-1 eluates (ASHF) is demonstrated in Table 15. At no dilution of the eluates or effluents was significant reactivity induced when PBS was substituted for KJ16-133 (not shown).

TABLE 21

Antigen-affinity purified and crude T cell supernatants contain T cell receptor $\beta$ chains

| | O.D. at 414 nm | | |
|---|---|---|---|
| | Antigen-Affinity Purified | | Crude |
| Final Dilution | Poly-18.68 ASHF• | Poly-18.68 ASHF(SF)+ | CPPD SN• |
| 1/10 | — | 0.759 | 0.287 |
| 1/100 | — | 0.287 | 0.162 |
| 1/500 | 0.677 | — | — |
| 1/1000 | 0.576 | — | — |

• Culture medium contained 10% FCS
+ Poly-18.68 hybridomas cultured in serum-free medium for ASHF production.

A 1/10 dilution of purified KJ16-133 antibody was added to wells coated with the indicated dilutions of Poly-18.68 ASHF preparations or crude supernatants (SN) from the T*h* line CPPD (See Tables 4 and 20). Protein concentrations of the preparations used for coating are: Poly 18.68 ASHF•-15 mg/ml; Poly 18.68 (SF)+ - 120µg/ml; CPPD SN - 1.5mg/ml. Conditions for the ELISA are described in Materials and Methods. The O.D. in uncoated wells reacted with KJ16-133 was -0.013. At no dilution of ASHF or CPPD SN was an O.D. of more than 0.056 when PBS was substituted for KJ16-133 (not shown).

The reactivity of T*h* lines and our ASHF preparations provide strong support for the hypothesis that ASHF are soluble forms of T cell receptors. The correlation of functional activity in CTLp induction by ASHF and reactivity with KJ16-133 in ELISA provide further support for this hypothesis. Immunoprecipitation studies of $^{35}$S-labelled material from Poly 18.68 and CPPD are in progress to determine the weight(s) of the molecular species recognized by KJ16-133.

In order to determine if the $\beta$ chains recognized by KJ16-133 are present on the moities responsible for ASHF activity in the affinity-purified preparations, a small column of KJ16-133 conjugated to Sepharose 4B was prepared. The results presented in Table 22 demonstrate that ASHF activity was detected in the original Poly-18.68 ASHF preparation and in the 2M NaCl eluate from the KJ16-133 column but not in the effluent that did not bind to the KJ16-133 column. In other experiments, CRBC-specific ASHF activity has been found in the 2M NaCl eluate fractions from samples applied to the KJ16-133 affinity column. The ability of the KJ16-133 column to bind ASHF activity indicates that the T*h*AR $\beta$ chain is one of the subunits of ASHF. The preparation of monoclonal antibodies that recognize T*h*AR $\alpha$ chains will be necessary to determine if these are other subunits of ASHF.

## D. Discussion

The results presented in this chapter provide strong support for the hypothesis that soluble forms of T*h* receptors are able to trigger CTLp in an antigen-specific manner. Specific binding of functional ASHF molecules to nominal antigen in the apparent absence of H-2-encoded molecules has been demonstrated previously in this thesis (Figure 3) and by other investigators (280-282). This is consistent with previous reports of binding of nominal antigen to T*h* in the apparent absence of H-2-encoded products (161-164).

Specificity in the recognition of antigen by ASHF and in the recognition of target cells by ASHF-induced CTL are confirmed (Tables 15-18). ASHF are only able to trigger CTLp in the presence of the antigen recognized by ASHF or the T*h* from which ASHF are derived.

## TABLE 22

KJ16-133 is able to deplete functional ASHF activity

| | % Lysis of CBA/J Targets | | |
|---|---|---|---|
| Dilution | Unfractionated Poly-18.68 ASHF | KJ16-133 Eluate | KJ16-133 Effluent |
| — | <1 | <1 | <1 |
| 1/50 | — | — | <1 |
| 1/100 | 19.0±4.7 | 7.1±2.9 | <1 |
| 1/200 | — | 14.6±4.9 | <1 |
| 1/400 | — | <1 | <1 |
| 1/500 | 10.7±5.3 | — | — |
| 1/1000 | <1 | — | — |

Unfractionated antigen (Poly 18)-purified ASHF was applied to an 0.5 ml KJ16-133-Sepharose 4B column, prepared as described in Materials and Methods (Chapter V). The column was washed with 5 ml PBS and the effluent was collected and concentrated by negative-pressure dialysis to the original volume of unfractionated ASHF. The column was eluted with 2M NaCl, dialyzed against PBS and concentrated to the original volume. One x $10^5$ BALB/c thymocytes were cultured with the plastic-adherent fraction from 3 x $10^5$ Poly-18 (1 μg)-pulsed (BALB/c x CBA/J) F1 stimulators and the indicated dilutions of unfractionated ASHF or KJ16-133 effluent or eluate fractions. Cytotoxicity induced against CBA/J targets was assayed on day 5. The % lysis induced by an optional concentration of IL2 (Fraction III) was 18.6 ± 4.2.

These types of specificity analyses at the induction and effector levels have been reported in many studies of the ASHF that participate in the induction of cell-mediated or antibody responses, as discussed at length in Chapter I. Criss-cross specificity analysis (Table 15) renders it unlikely that we have purified nonspecific lymphokines with differential affinity for carrier antigens.

The functional specificity of ASHF (Tables 15-18 and Chap. III & IV) clearly demonstrates that ASHF is distinct from antigen-nonspecific lymphokines such as IL2. Furthermore, it has been repeatedly demonstrated that ASHF preparations do not contain IL2 (Fig. 4, Ref. 239,294, and unpublished observations). The requirement of incomplete forms of ASHF for H-2-syngeneic adherent cells also demonstrates that it is functionally distinct from IL2 and CTDF (Tables 9,10, & 14). Our observations that CTLp require both ASHF and antigen for induction, and that the CTL induced are specific for the stimulator cell alloantigens (Tables 7 and 16), demonstrate that ASHF are not polyclonal activators and require recognition of antigen by CTLp for triggering. Results presented in this chapter also distinguish ASHF from IL2 by the requirement of ASHF for linked recognition in the induction of CTLp (Tables 17 - 19). Studies from the laboratory of H. Cantor (291) have demonstrated a similar requirement for linked recognition in ASHF-mediated induction of AFC responses. Tables 17-19 demonstrate that ASHF from LB19-1 and GK15-1 require intrastructural linkage of determinants recognized by ASHF and CTLp. CTLp are efficiently induced by either of these ASHF only when the determinants recognized by the ASHF (carrier determinants) and those recognized by CTLp (haptens) are presented in close physical proximity on the surface of the stimulator cells. The T*h*-derived signal required for the induction of CTLp is clearly distinct from the antigen-specific induction of IL2 release. Experiments demonstrating that in some experiments CTLp are inducible by ASHF or T*h* but not by IL2 (unpublished observations) provide further support for this hypothesis. It is suggested that under physiological conditions, only after antigen recognition and receipt of an ASHF-mediated signal, are CTLp able to be induced to proliferate and differentiate by antigen-nonspecific signals such as IL2 and CTDF.

In the absence of inhibitors of these antigen-nonspecific lymphokines *in vitro* a requirement for ASHF may be obscured.

Table 17 demonstrates that Poly 18.68 ASHF has requirements for physical association of hapten, carrier, and syngeneic (H-2-encoded) restriction elements on the same stimulator cell population. These requirements of Poly-18.68 ASHF are different from those of LB19-1 and GK15-1 in that the latter ASHF do not require presentation of the "carrier" determinants in the context of polymorphic H-2-encoded determinants. These experiments do not exclude the possibility that LB19-1 and GK15-1 ASHF require recognition of nominal antigen in the context of nonpolymorphic H-2-encoded determinants, for effective triggering of CTLp. The molecular geometry of the association of hapten, carrier, and MHC-encoded restriction elements that are required for interaction with soluble or membrane-bound forms of T*h*AR and CTR require further elucidation.

A requirement for linked recognition has been demonstrated in the induction of DTH and antibody responses (10-20,28). Experiments by Al-Adra and Pilarski demonstrate that T*s* also require linked recognition in the suppression of CTLp induction (348). The experiments presented in this chapter demonstrate the same requirement in the induction of CTL responses by ASHF. Linked or cognate (18) interactions are suggested to be primary requirements common to all classes of lymphocytes, that function to minimize the induction of "bystander" lymphocytes.

Figure 6 and Tables 21 and 22 indicate that our ASHF preparations contain $\beta$ chains of T cell antigen receptors, as determined by reactivity with the monoclonal antibody KJ16-133 (156). Strong reactivity of KJ16-133 with Poly 18.68 ASHF from serum-free and FCS-supplemented cultures and with LB19-1 (eluate) ASHF are demonstrated. Phenotypic analysis (Table 20) and mapping studies (J. Kappler, personal communication) indicate that KJ16-133 does not recognize C regions as previously claimed (158) but instead appears to recognize V, D, or J region determinants that are expressed at a relatively high frequency. The ability of a KJ16-133 antibody column to deplete ASHF activity from antigen-affinity purified

Poly-18.68 ASHF preparation clearly demonstrates that the functionally active moities in this preparation contain T*h*AR $\beta$ chains (Table 22). It is unclear if ASHF also contain $\alpha$ chains of T*h*AR. The development of other monoclonal antibodies recognizing C or V region determinants on T*h*AR chains will facilitate analysis of the molecular composition of ASHF. Analysis of mRNA from a variety of clones will enable testing for the possible existence of discrete membrane and secretory transcipts of $\alpha$ and or $\beta$ chain genes. Biochemical analysis of $\alpha$ and $\beta$ glycoprotein chains will be necessary to determine if secretory and membrane forms may differ due to translation of different species of mRNA and/or post-translational modification. It is reasonable to assume that both membrane and secreted forms of T*h*AR use the same or similar rearranged genes to generate variable regions that bind antigen. Comparison of the antigenic fine specificity required for the induction of IL2 secretion by Poly-18.68 hybridoma cells and for triggering of CTLp induction with Poly-18.68 ASHF will provide a functional test of the validity of this assumption.

In conclusion, the results of these studies suggest common and fundamental similarities in both cell-mediated and antibody-mediated immune responses. Different forms of clonally-expressed receptors on both T and B lymphocytes are suggested to function as membrane-bound antigen-recognition units or as soluble antigen-specific moities. A requirement for linked recognition the induction of AFC, DTH effector cells, and the T*h* that participate in the induction of DTH cells (10-20,28 and P. Bretscher, personal communication) as well as in the inhibition of CTLp by T*s* (348) has been demonstrated previously. The experiments presented in this thesis complete the picture by demonstrating that the induction of CTLp also exhibits this requirement for linked recognition.

The results presented in this thesis provide strong support for the previously described model of ASHF-mediated induction of CTLp (p. 35). It is suggested that complexes of T*h*AR and Ia antigens constitute primary inductive signals for CTLp, delivered or acting prior to the effects of nonspecific lymphokines that mediate maturation and clonal expansion. Both functional and molecular analyses support the validity of this model. The functional assay for

CTLp induction, developed in these studies, will enable further analysis of the role of ASHF in the triggering of CTLp. The adaptability of this system to accomodate almost any type of antigen will facilitate comparison of the mechanisms of CTLp induction with those of other classes of immune responses.

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